(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization
International Bureau

(43) International Publication Date
17 May 2001 (17.05.2001)

PCT

(10) International Publication Number
WO 01/34834 A2

(51) International Patent Classification[7]: C12Q 1/68

(21) International Application Number: PCT/DK00/00628

(22) International Filing Date:
10 November 2000 (10.11.2000)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:
PA 1999 01626 11 November 1999 (11.11.1999) DK

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(81) Designated States *(national)*: AE, AG, AL, AM, AT, AT (utility model), AU, AZ, BA, BB, BG, BR, BY, BZ, CA, CH, CN, CR, CU, CZ, CZ (utility model), DE, DE (utility model), DK, DK (utility model), DM, DZ, EE, EE (utility model), ES, FI, FI (utility model), GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KR (utility model), KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, MZ, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SK (utility model), SL, TJ, TM, TR, TT, TZ, UA, UG, US, UZ, VN, YU, ZA, ZW.

(84) Designated States *(regional)*: ARIPO patent (GH, GM, KE, LS, MW, MZ, SD, SL, SZ, TZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE, TR), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG).

Published:
— *Without international search report and to be republished upon receipt of that report.*

For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.

WO 01/34834 A2

(54) Title: ENDOGENOUS GENE EXPRESSION ASSAY

(57) Abstract: The present invention relates to a method for evaluating the possible physiological effects of a compound and/or a composition by assaying a cellular response thereto through measurement of changes in expression levels of at least one endogenous gene. The present invention provides new means to identify environmental chemicals or pharmaceutical compositions that interact with, amongst others, the endocrine system. The present invention thus relates to a method for detecting chemicals that present a health threat. Furthermore, the scope of the present invention includes means to investigate the specific response of an individual to a certain treatment as well as a cellular state of disease through assaying the expression level(s) of one or more endogenous marker gene(s) in a sample taken from such an individual. The results of such investigations may be used in diagnosis and in therapy.

Endogenous gene expression assay

FIELD OF THE INVENTION

The present invention relates to a method for evaluating the possible physiological effects of a compound by assaying a cellular response thereto through measurement of changes in expression levels of at least one endogenous gene. It is an object of the present invention to provide new means to identify environmental chemicals that interact with, amongst others, the endocrine system and thereby present a health threat. Furthermore, the scope of the present invention includes means to investigate a cellular state of disease through assaying the expression level(s) of one or more endogenous marker gene(s). The results of such investigations may be used in diagnosis of diseases.

BACKGROUND OF THE INVENTION

Scientific evidence suggests that humans and wildlife species may experience adverse health consequences from exposure to environmental chemicals that interact with the endocrine system. Reliable short-term assays that can be used to identify endocrine disrupting chemicals are needed to identify such chemicals.

The presence of endocrine disrupters in our environment has caused an increasing concern of their possible impact on wildlife and human health (Toppari et al.,1996). Investigators have focused on a possible decrease in human semen quality and an undisputed increased incidence of testicular cancer over the past few decades. These changes may be caused by intrauterine exposure of the male fetus to estrogens or antiandrogens (Sharpe et al., 1993). In addition, significant increases in the incidences of prostate-, endometrium- and breast cancer, and malformations of the external and internal genitals, such as hypospadia and cryptorchism, have been observed over the past 40-50 years, and may also be associated with increased exposure to estrogens (Henderson et al., 1982; Davis et al., 1993). Although still hypothetical, these malignancies may all originate from exposure to endocrine disrupters, especially estrogens (Henderson et al., 1982; Davis et al., 1993; Giwercman et al., 1992).

Furthermore, there are indications that other diseases, for example asthma and allergy, may be influenced or caused by similar environmental factors (Wist & Dold, 1997). It has been proposed that exposure to estrogens, or other hormones, is the cause for these

trends (Sharpe & Skakkebæk, 1993; Toppari et al., 1996; Kavlock et al., 1996; Cooper & Kavlock 1997). That estrogens are linked to malformations of the male genitals was in fact recognised as early as 1940 (Zuckerman, 1940) and that chemicals can have estrogenic properties was already shown for bisphenol A in 1936 (Dodds & Lawson, 1936).

In principle, any chemical that affects the endocrine systems is a potential endocrine disrupter, thus, endocrine disrupters include compounds that interfere with the estrogen, androgen and thyroid signalling systems. In addition, the many orphan receptors (reviewed in Blumberg & Evans, 1998) suggest that there may be other endocrine systems, and some of these may be targets for yet unknown endocrine disrupters. Presently, estrogens are by far the best studied compounds and most of the research into the mechanisms that are involved has also been performed on signalling through the estrogen receptors.

Environmental estrogens include a variety of very different chemicals such as polychlorinated biphenyls (PCBs), organochlorine pesticides, alkylphenols, phthalates, widely used preservatives such as parabens and food antioxidants (Toppari et al., 1996; Soto et al., 1995; Jobling et al., 1995; Zava et al., 1997). In addition, many plants and fungi contain compounds with estrogenic activity, the so-called phyto- and mycoestrogens (Toppari et al., 1996). Also, the potent synthetic hormones used for growth promotion in cattle in the USA and a few other countries should probably also be considered as potential endocrine disrupters. The large variability in the chemical structure of estrogenic compounds makes it impossible to predict their estrogenicity from the structure alone.

Hence, there is a strong need for reliable short-term methods that rapidly can detect chemicals with estrogenic properties. This is reflected in the ambitious Endocrine Disrupter Screening and Testing Program (EDSTP), that has recently been proposed by the US Environmental Protection Agency (EPA), where the EPA is concidering more than 87.000 substances as potential candidates for testing. These compounds include pesticides, commercial chemicals, ingredients in cosmetics, food additives, nutritional supplements and certain mixtures. The EDSTP is available online from EPA's website (http://www.epa.gov).

According to the EDSTP, the compounds will be tested for effects on the estrogen, androgen and thyroid systems in human, fish and wildlife, by a combination of *in vitro* and *in vivo* screening assays. The assays proposed by the EPA to detect estrogenic chemicals

include estrogen receptor binding assays (Taylor et al., 1984), transcriptional activation assays (Klein et al., 1994), rodent 3-day uterotrophic assay (Lan et al., 1976), rodent 20-day pubertal female assay (Gray et al., 1989) and fish gonadal recrudence assay (Sumpter et al., 1995). For chemicals that are positive in the screening, this will be followed by longer-term studies, to determine whether the chemicals cause adverse effects in humans, fish and wildlife and to establish a quantitative relationship between the dose and the adverse effect.

The validity and usefullness of some of the assays included in the EDSTP and other short-term estrogenicity assays was recently evaluated in a comparison study by Andersen *et al.* (Andersen et al., 1999).

Assays for estrogenicity are based on properties of the estrogen receptors. The *in vitro* assays utilise either its high affinity towards estrogens, or its function as an estrogen-dependent transcription factor and these *in vitro* assays are based either on measurement of direct binding to isolated receptors or on the induction of a reporter gene, regulated through the estrogen receptor (ER). ER binding assays involve the competition of the test compound with radiolabelled estradiol for specific binding to the ER in whole cells or in cell homogenate (Taylor et al., 1984). Alternatively, specific binding to isolated recombinant receptors could be measured (Andersen et al., 1999). The direct binding assay can easily be automated and thus scaled to accommodate testing of a large number of compounds. However, the estrogen receptor binding assay will only show how well the tested compound binds to the estrogen receptor, and does not define the ligand as an agonist or antagonist. Furthermore, chemicals that are metabolised to estrogenic compounds in mammalian cells are not detected in the cell free binding assays.

In reporter gene based assays, yeast or mammalian cells are transfected with the human estrogen receptor and a reporter gene such as β-galactosidase or luciferase, under the control of an estrogen responsive promoter. The activity of the reporter gene is directly related to the transcriptional activation activity of the test compound (Klein et al., 1994. The yeast based reporter gene assays can easily be automated, but they do not discriminate between estrogenic and antiestrogenic chemicals (Andersen et al., 1999). Mammalian reporter gene assays are under development, and they may eventually replace the current yeast based assays (Meyer et al., 1994; Vinggaard et al., 1999).

The *in vivo* assays are based on observations of phenotypical changes, for example increased uterus weight, that are initiated by estrogens, but like cell proliferation, phenotypical effects are always caused by complex changes. In animals, this involves many different cell types that probably respond differently to estrogens, some may respond by increased proliferation, other by recruiting other cells, etc.

In the rodent 3-day uterotrophic assay, estrogenicity is estimated as an increase in uterine tissue mass in ovariectomized or immature rodents after 3 days of treatment (Lan et al., 1976), and in the rodent 20-day pubertal female assay, estrogenicity is indicated by accelerated vaginal opening in weaning rats, after daily treatment from 21 days of age (Gray et al., 1989). Another commonly used *in vivo* estrogenicity assay measures the level of the yolk protein vitellogenin in male fish, which is very low in male fish, but increases in a dose-dependent manner after exposure to estrogenic compounds (Sumpler et al., 1995), and this is one of the primary endpoints in the fish gonadal recrudescence assay. The *in vivo* assays have several advantages, especially, they take into consideration the effects of metabolism, plasma-protein binding and pharmacokinetics, and typically cover a broader range of mechanisms of actions than *in vitro* assays. Because of their cost, complexity and ethical concerns, however, animal models are not suited for large scale screening of chemicals.

There are several attractive short-term assays that are not included in the EDSTP, including the cell proliferation assay (E-screen assay) by Soto *et al.* (Soto et al., 1995). The most "nature-like" *in vitro* assay, the E-screen assay, is currently the only *in vitro* assay that actually estimates cellular responses to estrogens, although induction of cell proliferation involves complex changes inside the cells, many of which are indirect effects of estrogens.

The E-screen assay is based on the dose-response relationship between the proliferation of human estrogen dependent breast cancer cells and the concentration of estrogen to which the cells are exposed, during six days of incubation (Soto et al., 1995). The sensitivity of the E-screen assay is relatively high, and it can discriminate between estrogen agonists and antagonists. However, the proliferative response is an indirect effect, and the assay is complicated by the toxicity of some compounds. Also, it is unclear to what degree proestrogens may, or may not, be activated to their estrogenic form in cultured cells.

In the clinic, Radio Immune Assay (RIA) is commonly used to measure serum estradiol concentration. Because it is antibody-based, RIA can only measure 17β-estradiol, although similar assays probably could be developed for other compounds. However, RIA assays have a detection limit of 10-20 pmol/L and cannot be used to measure for example the concentration of estradiol in the serum of prepubertal children.

Recently, Nishikawa *et al.* (Nishikawa et al., 1999) proposed an assay based on yeast-two-hybrid measurements of protein-protein interactions between ER and co-activators. Since estrogen agonist binding leads to the dissociation of co-repressors and recruitment of co-activators, this assay measures both the ability of a compound to bind the receptor and whether it recruits co-activators, and therefore it yields more information than a simple binding assay. Furthermore, other protein-protein interactions could be included, for example interactions with co-repressors could discriminate between agonists and antagonists because antagonists generally do not displace co-repressors.

The sensitivity of the currently available assays, as measured as the lowest detectable concentration of estradiol, is very different. The most sensitive is the MCF7 cell proliferation assay, where concentrations as low as 0.1 pM can be measured (Soto et al., 1995), and this assay may actually be able to measure concentrations in the femtomolar range. The yeast-based reporter gene assays are generally several orders of magnitude less sensitive, although measurements of very low estradiol concentrations have been reported (Klein et al., 1994). The fish vitellogenin assay can detect estradiol down to about 4 pM (Sumpler et al., 1995), whereas direct binding assays generally require concentrations in the nanomolar range (Andersen et al., 1999; Bolger et al., 1998).

However, estrogens function by interacting with the estrogen receptors, which are transcription factors that interact with many other signalling systems in the cells and organs, which then, in a co-ordinated manner, regulates the expression of target genes (Mangeldorf et al., 1995). Moreover, it has also been established, that the activity of the estrogen receptors is strongly affected by other signalling pathways, for example, the estrogen receptor-α (EP-α) can be activated by Epidermal Growth Factor (EGF), without estrogens present (Kato *et al.*, 1995). Moreover, differential expression of co-repressors and co-activators strongly affects the function of the ER, leading to large differences in the cellular responses to estrogens (Torchia et al., 1998). The complexity and synergy between sig-

nalling pathways is reflected in large cell-type-specific differences in the genes that are regulated by estrogens.

DESCRIPTION OF THE INVENTION

The present invention relates to methods for evaluating cellular responses to environmental compounds by determining or by comparing the expression levels of at least one endogenous gene.

The advantage of using an endogenous gene instead of importing an exogenous gene from a foreign genomic source, such as from prokaryotes in the form of plasmids comprising various reporter genes, is that the influence of the chemical compound is the single changed parameter and a true effect on the expression of the gene in its natural cellular environment is detected. Especially for receptor mediated interactions of the environmental compound, the use of an endogenous gene in its natural cellular environment is advantageous. Receptors often interact with other signalling systems in the cells, moreover differential expression of co-repressor and co-activators strongly affect the function of the receptor. In the case of using an exogenous reporter gene, co-ordinative effects may not be revealed, since a reporter gene assay only show how a particular, in most cases synthetic, promoter regulates the marker gene whereas the promoter of an endogenous gene includes all the elements necessary for the co-ordinated regulation of the gene.

In one embodiment this is achieved by comparing the environmental compounds' effect to the effect of other substances to the same sample, preferably to substances where the cellular response is previously known.

Endogenous gene expression assays can be used to evaluate cellular responses to any compound, provided that at least one gene that responds to that class of compounds has been identified. Thus, compounds acting through one or more of the androgen, thyroid, aromatic hydrocarbon, etc. receptors can be identified by determining the expression levels of at least one gene that is activated through one or more of these receptors. Especially, assays to detect effects caused by activation of ERβ and the androgen receptor can easily be developed from cell lines derived from the prostate.

Furthermore, endogenous gene expression assays are not limited to cell cultures, since changes in expression level(s) of selected gene(s) can be determined in different tissues from exposed animals. Especially, the number of animals required to assay thousands of compounds for their estrogenicity can be significantly reduced if gene-expression-related endpoints can be established. For example, when the specific gene(s) responsible for estrogen-induced uterus or breast growth has/have been identified and a relationship between its/their expression and a disease established, this gene or these genes can be assayed. An endpoint can then be: Fold induction of genes X and Y in tissue A and fold repression of gene Z in tissue B.

The present invention prescribes an assay to directly analyse human samples, for example derived from blood or any other tissue. This requires identification of the gene(s), expressed in blood cells, that is/are affected by exposure to a particular environmental compound, e.g. a putative endocrine disrupter compound.

In the present application "environmental compound" is defined as any compound in the environment that may enter the body by ingestion, absorption through the skin or by inhalation or otherwise taken into the body, examples of "environmental compounds" are all components of food, cosmetics, hygiene products, pharmaceuticals and smoke.

"Endogenous gene" is herein defined as a gene selected from the group consisting of all the genes included in the genomes of any mammalian or fish that are expressed at any developmental stage of the mammal as opposed to an exogenous reporter gene construct artificially introduced into a cell or animal.

"Cellular response" is herein defined as a change in gene expression of at least one endogenous gene or a post-translational modification of an already expressed gene product, the change in gene expression is understood as a change in the level of gene expression, or post-transcriptional modification of the RNA products, post-translational modifications are understood as changes in both the primary structure of the polypeptide and any other modification of the protein, the following examples which are meant to be illustrative and not limiting are phosphorylation, ADP-ribosylation and glycosylation.

"Evaluate" is herein defined as any method that can quantify gene expression on either RNA or protein level or both, e.g. by PCR based methods including competitive PCR,

hybridisation based methods including DNA chip array, 1D and 2D-gelelectrophoresis, mass spectrometry, immuno-chemistry and SAGE (Serial Analysis of Gene Expression).

"Quantify" is herein defined as determining the amount of label incorporated into a DNA fragment by PCR or during PCR amplification (including labelled primers and nucleotides) by any method known.

One embodiment of the present invention is the usage of DDRT-PCR technology which allows quantification and comparison of the expression levels of given mRNAs among different samples, where the ratio between any two samples will show the difference in expression level of the mRNA in the two samples. To visualise the differentially expressed genes amplified by the PCR, a detectable label, e.g. a radioactive isotope, fluorescent group or biotin is incorporated.

In the case of using radioactive labelling, quantification of the expression level by Phosphor Imaging scanning further introduces an advantage by producing a very reproducible result and thus accurate estimates of activity of the compound.

If fluorescent labels are used the results can be analysed on a DNA sequenator. Fluorescent labels reduce the sensitivity by 10 to 100 fold, although it may be possible to optimise the PCR and the detection and thereby obtain a sensitivity that is close to radioactivity based assays. Especially, the Taq-Man technology (Perkin-Elmer) may be an attractive alternative. Another possibility is the use of DNA chip technology (Bowtell et al., 1999; Debouck et al., 1999). For example, construction of a chip with e.g. 100 regulated genes and a similar number of unaffected genes allows almost complete automation of the assay.

One field of environmental compounds which are highly relevant for the experienced adverse health consequences are chemicals that interact with the endocrine system. In principle, any chemical that affects an endocrine system is a potential endocrine disrupter, thus, endocrine disrupters include compounds that interfere with the estrogen, androgen and/or thyroid signalling systems. In addition, the many orphan receptors (reviewed in Blumberg & Evans, 1998) suggest that there may be other endocrine systems, and some of these may be targets for yet unknown endocrine disrupters. Presently, estrogens are by

far the best studied compounds and most of the research into the mechanisms that are involved has also been performed on signalling through the estrogen receptors.

Although the estrogen receptor is a potent transcription factor, only a few assays are actually based on measurements of direct receptor-mediated effects of estrogens on the expression of genes. Presently, these assays only include marker gene based assays, such as the yeast E-screen (Klein et al., 1994) and different marker gene constructs in mammalian cells (Bonefeld Jørgensen et al., 1997; Vinggaard et al., 1999). These assays have in common that they utilise artificial responsive constructs and they may therefore not properly reflect estrogen-induced changes in the expression levels of endogenous estrogen-regulated genes that, in both cultured cells and *in vivo*, are directly responsible for the phenotypic responses.

The assay of the present invention quantifies estrogen-induced changes in the expression of endogenous genes, either in cultured cells, experimental animals, or directly in humans. Such an assay is designed to evaluate the estrogenicity of a compound by quantitfying changes in the levels of genes directly regulated by estrogens in cell cultures or animals. Furthermore, the endogenous gene expression assay is designed to evaluate changes in expression levels of the genes that are causing the adverse effects of the investigated type of compounds.

Any animal species or cell line can be used in the endogenous gene expression assay, provided it responds to the compound that should be tested. Thus, it is important first to precisely define what the assay should detect. If the aim is to establish an endogenous gene expression assay that can be used to screen compounds for estrogenic properties, the most appropriate is a cell-culture-based assay, since this is more flexible, much faster and much less expensive than an animal-based assay. However, because of the complexity of the endocrine system, endocrine disrupter screening must also include animal models. Therefore, the most sensitive, and the most "human-like" animal model must be chosen, while still keeping the costs and potential ethical problems in mind. In most cases, a rodent model will be the most realistic. However, different laboratory mice strains exhibit a very large variability in their sensitivity towards estrogens and this is probably also the case for other animals (Spearow et al., 1999). Thus, it is necessary to investigate whether the chosen species, and the specific strain, is sensitive to low concentrations of estrogens. Next, the most sensitive, or the most severely affected, tissue must be identi-

fied and it must be established in which developmental stage the adverse effects occur, i.e. should the animals be exposed *in utero* or as adult animals?

The present invention demonstrates that the estrogenic activity of a compound can be evaluated by assaying induction or repression of endogenous estrogen-regulated "marker genes" in human breast cancer MCF7 cells. Four marker genes were included in the assay: pS2, transforming growth factor β3, monoamine oxidase A and α1-antichymotrypsin, and estrogenic activity was evaluated for 17β-estradiol, diethylstilbestrol, α-zearalanol, nonylphenol, genistein, methoxychlor, endosulphan, o,p-DDE, bisphenol A, dibutylphthlate, 4-hydroxy tamoxifen and ICI 182.780. All four marker genes responded strongly to the three high-potency estrogens (17β-estradiol, diethylstilbestrol and α-zearalanol), whereas the potency of the other chemicals was 10^3-10^6 fold lower than that of 17β-estradiol. There were some marker-gene-dependent differences in the relative potencies of the tested chemicals. TGFβ3 was equally sensitive to the three high-potency estrogens, whereas the sensitivity to α-zearalanol was approximately 10-fold lower than the sensitivity to 17β-estradiol and diethylstilbestrol when assayed with the other three marker genes. The potency of nonylphenol was equal to that of genistein when assayed with pS2 and TGFβ3, but 10-100 fold higher/lower with monoamine oxidase A and α1-antichymotrypsin, respectively. The results are in agreement with results obtained by other methods and suggest that an assay based on endogenous gene expression offers an attractive alternative to other E-Screen methods.

The present invention shows that the estrogenic activity of a chemical can be evaluated by assaying induction or repression of at least one endogenous estrogen-regulated "marker gene" in human estrogen-dependent breast cancer cells. It also shows that changes in gene expression levels quantitatively show a dose/response correlation.

Comparison of expression levels determined by PCR is a well characterised method, that is used in a variety of protocols. This has established that PCR amplification of cDNA fragments conserves the relative levels as they were in the original mRNA preparations, provided proper manuals are used (Orlando et al., 1998). Thus, the level of an amplified radioactively labelled PCR fragment is directly proportional to the level of the corresponding mRNA in the original sample. The low-stringency PCR method described in the present application is, in principle, similar to multiplex PCR, where a few DNA fragments are amplified in the same reaction, although multiplexing in the present invention is ob-

tained using a single primer pair and low-stringency annealing. The result is an amplification in average about 125 different PCR fragments in each reaction. Because the competing fragments are identical in all samples prepared with a given primer pair, only the level of the estrogen-sensitive mRNA/cDNA fragment will change, and the intensity of the corresponding band accurately reflects the level of the mRNA in the RNA samples. However, in contrast to e. g. Northern blotting, the precise amount of an mRNA is not reflected in the intensity of the band. The DDRT-PCR technology allows comparison of the expression level of a given mRNA among different samples, where the ratio between any two samples will show the difference in expression level of the mRNA in the two samples.

In the examples, the expression levels are quantified by Phosphor Imaging scanning, and normalised according to either a constant band or the background, resulting in very reproducible results and thus accurate estimates of estrogenic activity. This is shown by the very small variation introduced in the cDNA synthesis and PCR (24% and 16%, respectively), compared to the many fold induction or repression resulting from exposure to the estrogenic compounds. Although other quantification methods may be used, Phosphor Imaging scanning is therefore presently preferred.

An alternative assay can be based on quantification of estrogen induced changes in the expression levels of one or more endogenous genes, either in cultured cells or in selected tissues from exposed animals. With the endogenous gene expression assay of the present invention, it is possible to assay for induction of genes that are known to be regulated by different signalling pathways as well as for genes that are regulated directly by the estrogen receptor. Compared to this, a reporter gene assay will only reveal how a single gene/promoter is regulated, and, for example, effects caused by cross talk between different signalling pathways may not be detected. In addition, assaying gene expression in several tissues from exposed animals ensures that effects derived both from the test compound and from its metabolites are detected, including tissue specific effects. In the end, all the endpoints currently used to determine estrogenicity in animals (and cells) are derived from changes in gene expression, and this, changes in the expression of endogenous gene(s), could be as good an endpoint as, for example, increased uterus weight, provided the responsible gene(s) have been identified.

The examples of the present invention show that a cell based endogenous gene expression assay is very sensitive, and that it can be used to assay the estrogenicity of different

putative estrogenic chemicals (Fig. 1). In the examples, estrogenicity is evaluated as induction or repression of four endogenous estrogen-regulated "marker genes" selected from a collection of previously identified estrogen regulated genes. The assay is performed in human estrogen-dependent breast cancer MCF7 cells, and changes in gene expression are assayed by a competitive PCR method and displayed on PAGE gels as for Differential Display of Reverse Transcribed mRNAs technology (DDRT-PCR) (Liang et al., 1992). The expression levels are subsequently quantified by Phosphor Imaging.

However, the person of ordinary skill in the art will appreciate that the results can be extrapolated to be used in a broader context: First an appropriate model for the endogenous gene expression assay is chosen and it is established how and when the cells or animals should be exposed to the chemical(s) and which tissues should be analysed, and appropriate marker genes are selected. If responsive genes already have been described in the literature, it is generally recommendable to first investigate the response of these genes because this will show whether the cells or tissue responded as expected. However, there are many reports of estrogen-responsive genes that cannot be reproduced, either because they have been defined as estrogen-responsive although their regulation is, in fact, a secondary effect of estrogen, this includes, for example, many proliferation sensitive genes; or because of laboratory-specific differences among strains and cell lines. Thus, in most cases it is necessary to perform a random screening for responsive genes. In principle, this is done by comparing gene expression in exposed versus non-exposed samples and any differential gene expression screening technology can in principle be used. However, it is important that the chosen technology actually can detect differentially expressed genes, also when the difference in expression levels is "only" a few fold. Thus, technologies that mainly detect "on-off" regulation, where the expression of a gene must essentially be zero in one sample and very high in the other before it is detected, should be avoided, because very few estrogen-regulated genes show this type of regulation. The most widely used technology is Differential Display of Reverse Transcribed mRNAs by PCR (DDRT-PCR) (Liang & Pardee, 1992), but other technologies such as Serial Analysis of Gene Expression (SAGE) (Velculescu et al., 1995) or gene arrays (Bowtell, 1999) could also be used.

If the test system is a cell line, screening for differentially expressed genes is straight forward; gene expression in cells grown in the absence and presence of the test compound is compared and differentially expressed "bands" are sequenced to identify the corre-

sponding mRNAs. Moreover, effects of different estrogens can also be analysed during a screening for affected genes (Fig. 2). In a cell line, most bands (mRNAs) will be constant; based on analysis of more than 40,000 bands, estimably less than two hundred mRNAs are affected by estrogens in human MCF7 cells. Thus, most of the bands will be constant, greatly facilitating detection of differentially expressed bands.

Screening for differentially expressed genes in an animal model is more complicated, because multiple organs and developmental stages must be included. An example of a DDRT-PCR analysis of gene expression in mice testes during foetal development is shown in Fig. 3. In contrast to a cell line, gene expression in a developing organ is highly time-dependent, since the expression of most mRNAs change during embryogenesis, and mRNAs that are specific for the different stages can easily be identified (Fig. 3). However, even though it is known that the testis is affected by exposure to, for example, diethylstilbestrol (DES) during embryogenesis (Stillman, 1982), it is not know at which stage the effects occur. Therefore it is necessary to perform a time-course study and a number of samples must be compared. In the study illustrated in Fig. 3, samples were prepared at 16 different time points from both DES exposed and non-exposed animals, and each primer combination analysis thus includes 32 samples. Although this complicates the screening, it does not require undue experimentation to detect differentially expressed genes.

The marker genes.

In the present invention, estrogenicity is determined by analysing the expression levels of four marker genes: pS2, MAO-A, TGFβ3 and α1-ACT. Since the assay is based on endogenous gene expression an arbitrary number of marker genes could, in principle, be included in the assay, and the marker genes used in this invention could be replaced by other estrogen responsive genes, such as the progesterone receptor or any other gene from the following list of genes that are identified as potentially regulated by estrogens:

List of genes

Identity	**Accession No.**
Alpha-tropomyosin	M19714
Prothymosin alpha	M14630
Calnexin	L18887; M94859
Plasma membrane Ca2+ ATPase	J04027

Alpha-1 antichymotrypsin	J05176
Nrf2 (NF-E2-like basic leucine zipper)	S74017
H+-ATP synthase subunit b	X60221
RAN, GTP binding nuclear protein	N54562
CoxVb (cytochrome c oxidase Vb)	M59250
NAD(P)H:menadione oxidoreductase	J03934
GSTM3 (glutathione transferase mu 3)	J05459
GSTM4 (glutathione transferase mu 4)	M96233
Ribosomal protein s9 (RPS9)	U14971
NADH-ubiquinone oxidoreductase	
B8	N36041
Estrogen receptor alpha	M12075
Mrg1	Y15163
Monoamine oxidase A	X60819
14-3-3 beta	X57346
Nrf1 (NF-E2-like basic leucine zipper)	L24123
Ezrin-radixin-moesin binding	
phospho protein	AF015926
NAD-dependent methylene	
tetrahydrofolate dehydrogenase	X16396
General transcription factor IIB	X59268
Cytochrome P450 1B1 (CYP1B1)	U56438
Voltage dependent anion channel 1	L06132
Na,K-ATPase beta (ATP1B)	M25160
Helicase-like transcription factor	Z46606
PS2	X00474
c-myb	M13665
Dolichol phosphate mannose syn-	D86198

thase	
Transforming growth factor beta3	
(TGF beta3)	X14891
Splicing factor Srp40-1, Srp40-3	U30827, U30826
Stimulator of TAR	U38846, AF026291
Superoxide dismutase (SOD)	L44746
Krueppel-related DNA binding protein	M61871
Rap1a GEF	AF081195
X104	L27476
KIAA0177	D79999
KIAA0228	D86981
KIAA0324	AB002322
KIAA0587	AB011159
EST, GLYT-1-like	AA541466
EST	D12089
EST	H75980; R16640; H03983; M85486 ect.
EST	H22521; T29588; W68746; M22632 ect.
pLiv-1	U41060
EST	F05012; F02632; F01656; Z40675
EST	N28895
BBC1	X64707
EST	X64889; X16064; W52771; L13806 ect.
EST	R19672; T80223; F06854; F05628 ect.
EST	AA147794; N26750; N29917; F01346 ect.
EST	AF011889; AC002480; AC003670; AC003986
EST	T34308; W48733; AA286770; AA565818 ect.
EST	F17401; F19592; F16329; F16791 ect.
EST	AA133702; AA181060; AA195993; AA534174 ect.
EST	W23587; N66155; T40877; G14503 ect.
EST	W51846; AA194088; N98678; AA235211 ect.
EST	R86934; H56044
EST	N33955; R31346; R27860

EST	R72982
EST	F17917; D52124; F18552; F19053 ect.
EST	H00745; N64553; N23747
EST	D79753
EST	F15988; F16027; F15994; F16255 ect.
EST	D63875; E08855
EST	W56184; C21477; AA604229; AA632369 ect.
EST	AA148404; AA782756; AA431739; TO3882
EST	AA677552; AA731207

PS2. The expression level of the pS2 mRNA is a widely used indicator of estrogenicity and the translation product of the pS2 mRNA is also induced in MCF-7 cells in response to estrogen (Jakowlew et al., 1984). The 9kDa encoded protein belongs to the trefoil family of peptides, whose members probably are involved in regulation of proliferation since it has been shown that trefoil proteins activate the Ras/MEK/MAP-kinase signal transduction pathway by direct interaction with EGF receptors. There are no reports in the literature of non-estrogenic compounds that induce pS2 expression in MCF7 cells.

The human pS2 gene (acc. no. X05030) (Jeltsch et al., 1987) contains an imperfect Estrogen Response Element (ERE) that varies from the consensus palindromic ERE (GGTCAnnnTGACC) by one base pair in its right arm (GGTCACGGTGGCC). Functional imperfect EREs have been demonstrated in a number of estrogen responsive genes, including the human TGFα, human cathepsin D, the rat progesterone receptor and the Xenopus laevis vitellogenin gene and are most likely responsible for the observed estrogen-dependent regulation.

For example, expression of the pS2 gene in human breast cancer cell lines is variable. In MCF7 cells, pS2 is one of the major estrogen-regulated genes (Jakowlew et al., 1984), whereas it apparently is not expressed in another estrogen-dependent breast cancer cell line T47D (Fig. 4) (Nutt et al., 1991).

MAO-A. The MAO-A gene encodes one of the two monoamine oxidase proteins (A). The expression and activity of MAO genes (A & B) have been investigated both *in vivo* and in *vitro* (Holschneider et al., 1998). *In vivo* assays have been conducted because there seems to be a correlation between mood changes and estradiol levels that may be medi-

ated through the activity of monoamine oxidases (Klaiber et al., 1996). The activity and expression of MAO-A is both *in vivo* and *in vitro* inversely correlated to the estradiol concentration (Holschneider et al., 1998), similar to the regulation we have observed in MCF7 cells. However, MAO genes may be induced by E2 in some tissues, and Sarabia and Liehr (Sarabia and Liehr et al., 1998) have shown that expression of the MAO-B gene actually is induced by E2 in hamster kidneys.

The human MAO-A gene (acc. no. AL020990) contains several putative ERE sequences, that could be responsible for the observed estrogen-dependent regulation of the MAO gene. One is located 200 bp upstream from the transcription initiation site, and differs from the consensus palindromic ERE by two base pairs in its right arm (GGTCACCTTCCC). Moreover, the two half-palindromic sequences are separated by only two base pairs. Another putative imperfect ERE is located approximately 1500 base pairs upstream from the transcription initiation site and contains a single mismatch in the left arm (GGACAAATGTGACC), and four base pairs between the two half-palindromic sequences.

TGFβ3. Repression of TGFβ3 expression by estradiol in MCF7 cells has previously been described and cell- and tissue-type-specific expression has also been shown (Takahashi et al., 1994). TGFβ3 expression is regulated by non-estrogenic compounds and it is probably necessary to include additional marker genes to be sure only to detect estrogens. It has previously been reported, that TGFβ3 inhibits the growth of MCF7 cells and that secretion of TGFβ3 is induced by anti-estrogens. In our hands, however, TGFβ3 was only slightly increased after exposure to a high concentration of 4-OH-TAM (Fig. 5).

The human TGFβ3 sequence (acc. no. X14885) contains a putative imperfect ERE 340 bp upstream from the transcription initiation site, that varies from the consensus ERE sequence at two positions, one in each half-palindromic sequence (GGCCAGCAACTGCCC). Also, the two half-palindromic sequences are spaced by 5 base pairs, instead of 3.

α1-ACT. Estrogen-mediated induction has previously been described for the antiprotease α1-antichymotrypsin in human breast cancer cells (Massot et al., 1985).

The human α1-ACT gene (acc. no. AL049839) contains a putative ERE sequence approximately 570 base pairs upstream from the transcription initiation site, however, as for TGFβ3, the ERE differs from the consensus sequence at two positions, one in each arm (AGTCACTGTGGCC).

Other genes could be included in the assay, including genes regulated by either ERα or β, together with genes regulated by other pathways. Also newly identified receptors, such as SXR and PXR *(reviewed in* Blumberg et al., 1998), that are activated by a variety of different compounds, including estrogen antagonists and agonists, could be involved in the activation/repression of some of the estrogen-sensitive genes identified. The reversal of estrogen induced changes by ICI 182.780 observed for the four marker genes, however, makes it unlikely that they are regulated through these new receptors.

It should be noted that "marker-genes" often are highly cell type specific. For example, MAO-A and TGFβ3 were expressed in the estrogen-dependent breast cancer cell line T47D, and both were repressed by estrogens, whereas pS2 was undetectable and the expression level of α1-ACT was very low, much below that in MCF7 cells, although it probably also is induced by estrogen in T47D. Thus, the diverse action of estrogens on different tissues and cell types is reflected in the genes that are estrogen-regulated in the different cells, suggesting that cell-specific differences in gene expression maybe can be exploited for the detection of new SERMs.

Currently blood samples are analysed for the expression of the E2 regulated genes that were identified in the screening, and preliminary results show that many are expressed in cells in the blood. However, the expression of one of the genes, monoamine oxidase A, in white blood cells is not correlated with the total E2 levels in the serum. This is probably caused by inter individual differences in the level of SHBG, that binds, and inactivates, 98% of the serum E2.

However, the intent of the invention is not to correlate gene expression and serum E2 levels, but to compare levels in persons exposed to endocrine disrupters with non-exposed persons, or to the same person before and after the exposure, and SHBG does not interfere with such comparisons because SHBG does not bind these compounds. Furthermore, the endocrine activity of the endocrine disrupters will be in addition to the effects of

the natural hormones and their activity can thus easily be detected as an increased or decreased gene expression level, as compared to before and after the exposure.

One embodiment of the invention relates to the analysis of blood samples for expression of the E2 regulated genes that were identified in the screening mentioned above. It is shown that many of the genes that are regulated by E2 in MCF7 cells, also are expressed in various cells present in blood. However, at present there is no information concerning possible E2 regulation in the blood, although reports in the literature suggest that there are E2 regulated genes in blood and that for example, MAO-A may be a candidate for an E2 regulated gene that can be assayed in human blood samples.

Establishment of an endogenous-gene-expression assay.

When a candidate marker gene has been identified, it must be tested for its usefulness in an assay system. The main conditions are: 1) its expression should be highly sensitive to estrogens, i.e. it must respond to very low concentrations; 2) it must show a strong dose-dependence in its expression level and; 3) the difference in expression levels in exposed and non-exposed samples must be relatively large, at least several fold.

Thus, when one or more potential marker genes have been identified, it/they must be tested in dose-response type experiments (Fig. 6) to analyse how sensitive it is/they are to the test compound and also whether its/their expression levels correlate with the hormone concentrations in the samples (Fig. 6B). Based on the results of these experiments, the best 4 or 5 genes are selected and further tested for their response to previously characterised estrogenic compounds, to ensure that all the already known estrogens are detected by the assay (Fig. 3). Provided these tests are successfully completed, the selected genes can most likely be used to assay for estrogenicity.

Different compounds have different potencies on different genes.

In the present invention, estrogenic activity was demonstrated for all the putative estrogenic test compounds, and the results correlate well with previous reports (Soto et al., 1995; Zava et al., 1997; Andersen et al., 1999). Furthermore, due to conversion to estrogens, 10^{-7} M testosterone had an estrogen-like effect on the expression of the marker genes, whereas 10^{-7} M progesterone had no effect on the expression levels.

The four marker genes chosen all responded strongly to the high-potency compounds (E2, DES and ZA) but there were some differences in their potency, defined as the minimum concentration required to affect the expression of a marker gene. The potency of E2 and DES was similar when assayed with pS2, TGFβ3, and α1-ACT whereas MAO-A seems to be more sensitive to E2 than to DES. The potency of ZA was equal to that of E2 and DES if assayed with TGFβ3, but more than 10-fold lower than E2 and DES for the other three marker genes.

The potency of the environmental estrogens was much lower than that of E2, DES and ZA. As for the high-potency compounds, there were some differences in the relative potency of the low-potency compounds on the four genes (Table 2). For example, the potency of NP was equal to the potency of GS on two marker genes (pS2 and TGFβ3), but 10-100 fold higher/lower, respectively, when assayed with MAO-A and α1-ACT.

The relative potency of the compounds generally correlated with the relative change in expression level of the four genes, although this was most evident for pS2. For example, the expression level of pS2 was induced almost 25-fold after exposure to 10^{-10}M E2, approximately 16-fold after exposure to 10^{-6}M GS and less than 10-fold after exposure to 10^{-4} M DBP. For the other marker genes, this trend was less consistent. It should be noted that the fold induction / repression is very sensitive to the initial levels, and a slightly elevated level in the non-induced cells will lead to significant variation in the "fold change". Thus, the precise fold-change varies between experiments, whereas the relative differences between compounds are maintained.

High concentrations (10^{-8} - 10^{-6} M) of TAM-OH seems to have a small but detectable E2-like effect on the expression of MAO-A, pS2 and α1-ACT, whereas similar concentrations may have a small stimulatory effect on TGFβ3. However, the required concentrations are so high that the effects may not be ER-mediated, similar to what has been observed in SK-N-BE / SK-ER3 cells, where the observed effects of TAM apparently are independent of the presence of ERα. Unfortunately, the possible expression of ERβ has not been investigated in the SK-N-BE cells, although the lack of E2 response indicate that neither receptor subtype is present.

During the screening for estrogen-regulated genes, several related genes were detected that were consistently more sensitive to DES and ZA than to E2. These genes, however,

show a time-dependent expression profile that is different from the majority of the identified E2 regulated genes, since their expression was not affected before 8-16h exposure to the hormones, whereas almost all other E2 regulated genes, including the four marker genes, were affected within 2-8h.

Person-to-person variation in the sensitivity to the assayed compounds are detected by a "gene expression level analysis", this shows the actual response in the individuals. This is important, since there are very large genetic differences in the response to estrogens (Spearow et al., 1999), and most likely also to other compounds.

For example, there are significant differences in the expression levels of the dioxin receptor (Hayashi et al., 1994), which results in differences in the sensitivity to dioxins. The observed genetic variation suggests that humans should be tested for their sensitivity to putative compounds before they are administered. For example, the individual should be tested for his or her sensitivity to estrogens before administering birth control pills or hormone replacement therapy. Currently this is not possible, but the technology described in the present invention assays exactly that.

Methods for quantifying expression levels.

When appropriate marker gene(s) have been identified, essentially any method that precisely can quantify their expression levels in different samples can be used in the assay. In the present invention, a competitive PCR method has been used, and the results are displayed as for Differential Display, followed by quantification of the bands corresponding to the marker genes. However, this method is probably not the best for large scale use, since it requires specially trained personal and it is not a method that easily can be used in, for example, the clinic. Thus, other methods must be considered and one of the best candidates is the emerging DNA-chip, or other DNA-array, technology (Bowtell, 1999; Debouck & Goodfellow, 1999). This technology is based on immobilising different cDNAs on a solid support, and the expression of the corresponding genes in different samples is compared by hybridising labelled cDNA from each sample to a chip/array. The intensity of the hybridisation signal, on chips/arrays that have been hybridised to labelled cDNAs from the different samples, is then determined, similar to the quantification of bands displayed on a gel. This method will eventually become the method of choice, however, at present it is not as sensitive as the PCR-based method outlined above. The person of skill in the art

will be aware of other technologies that may be used, such as the Taq-Man system from Perkin-Elmer and similar PCR-based semi-automated methods.

By the establishment of a rapid and sensitive technology for determining expression levels of endogenous genes has been established, the expression of any gene can be monitored, opening for a general use of endogenous-gene-expression-assays in a variety of different systems. For example, the identification of the gene(s) responsible for malignant transformation of breast cells, leading to breast cancer, can form the basis for a knowledge-based assay. The induction or repression of these genes can be an "end point" in a toxicological analysis, where the criteria for rejecting a compound could be: It induces genes A, B and C more than two fold in tissue X, and it reduces the expression of genes D and E to less than 50% in tissue Y. Such assays will be far more informative than the currently used assays and will significantly reduce the number of animals required for the testing, because it could be expected that essentially all the animals in a test group will respond to the compound. However, only a few will actually develop cancer, because this does not only depend on its initiation; additional events are required before the animal gets cancer. The same principles could be adopted in other toxicological analyses, the only condition is that the it must be analysed which genes are relevant to use in the particular analysis.

The ultimate endocrine disrupter screening system should be performed in humans, or rather, the endocrine disrupter screening technology should be able to monitor whether a person has been exposed to an endocrine disrupter. However, for ethical reasons, humans cannot be used as test animals and therefore different doses of potential dangerous chemicals cannot be administered to human test subjects. Thus, a screening for endogenous marker genes would be difficult to perform on human subjects. Alternatively, the expression levels of estrogen-regulated genes, that originally have been identified in a human cell line or by other methods, can be analysed in human primary cell cultures or blood samples (Fig. 7). Unfortunately, most of the estrogen-regulated genes in human MCF7 cells, that were tested as markers in human blood samples, are either not expressed in blood cells, or their expression levels do not correlate with the measured serum estradiol concentrations (Fig. 7). However, since about 98% of the measured serum estradiol is bound to SHBG and thus inactive, small variations in the SHBG levels may have large effects on the amount of free estradiol. This could be the reason for the apparent lack of correlation between expression levels and estradiol concentrations. Most likely,

there are estrogen-regulated genes present in blood cells, and it will be possible to monitor "total estrogen load" by analysing gene expression in human blood samples.

A rather surprising aspect of using endogenous genes for determining estrogenicity is, that genes apparently have differential sensitivity towards different estrogens. In MCF7 cells, monoamine oxidase A, for example, is more sensitive to nonylphenol than to genistein, whereas α1-antichrymotrypsin is more sensitive to genistein than to nonylphenol; for some genes, the difference in sensitivity is about two orders of magnitude. These differences could be the reason why exposure to different compounds, with similar estrogenic potencies, results in different adverse effects, exemplified by the differential sensitivity of the male and female reproductive tract to 17β-estradiol and DES (e.g. Khan et al., 1998). Thus, endogenous gene expression assays may reveal differences between estrogens that are not apparent from other assays.

EXAMPLES

Example 1

Adjustment to "estrogen-free" cell culture conditions.

The described endogenous gene based assay was developed because a strong correlation between the expression of some genes and the concentration of estrogen in the cell media was observed in a screening for estrogen-regulated genes. This suggested that expression of endogenous genes could be an useful method for assaying the estrogenicity of different compounds, and that different genes could respond differently to different estrogens. To evaluate whether endogenous gene expression could be used to assay compounds for potential estrogen activity the different steps in the procedure were optimised and the estrogenicity of a range of compounds tested.

To determine the time needed for the cells to adjust their gene expression to "estrogen-free" conditions, MCF7 cells, cultured in a standard DMEM medium, were transferred to an estrogen free medium and RNA was harvested on 6 consecutive days. The expression level of the estrogen inducible pS2 mRNA, a widely used indicator of estrogenicity, was evaluated by DDRT-PCR using a targeted upstream primer (see below). This showed that the level of pS2 mRNA expression was relatively high when the cells were cultured in the standard DMEM medium and decreased in a time dependent manner after incubation in

the "estrogen free" medium (Fig. 8). After 3-4 days, the expression level of pS2 was reduced sufficiently to perform the experiments. However, to ensure that the cells had adapted completely to estrogen-free conditions, all experiments are made on cells that were incubated for at least 6 days in estrogen free medium.

Cell culturing and hormone exposure.

To avoid any sample-to-sample contamination, all cells used in this study were cultured in 25cm^2 flasks. Human estradiol-dependent MCF-7 breast cancer cells (a kind gift from Dr. P. Briand, The Danish Cancer Society, Copenhagen, Denmark) were grown in a DMEM medium (Gibco BRL) containing 5 % foetal bovine serum (FBS) (Gibco BRL), 1 nM insulin (Boehringer Mannheim), 2 mM L-glutamine (Gibco BRL), 1x non essential amino acids (Gibco BRL) and 25 IU/ml Penicillin-Streptomycin (Gibco BRL). The medium was changed every two-three days. Six days prior to addition of the test compounds, the cells were washed in PBS (Gibco BRL), and the medium was substituted with a DMEM medium without phenol red (Gibco BRL), containing 5 % dextran-charcoal stripped FBS, and otherwise as the standard medium described above. Steroids were removed from FBS: FBS was incubated with 0.5 % activated charcoal (Sigma) and 0.05 % dextran T-70 (Pharmacia Biotech) for 30 min at 55°C, and the charcoal particles were removed by centrifugation at 4°C for 20 min at 4500× g. This step was repeated, and the stripped serum was sterile filtered and stored in aliquots at - 20°C. 40-50% confluent cells were rinsed in PBS and fresh medium containing the respective test compound was added. The control cells received only the vehicle (ethanol).

Example 2

Screening for Estrogen-regulated Genes in MCF7 Cells.

The use of endogenous genes and the DDRT-PCR technology to assay for estrogenicity implies the prior identification of estrogen-regulated bands, corresponding to potential marker genes, on DDRT-PCR gels. Hence, MCF7 cells were screened that were either unexposed or exposed to 10^{-8}M 17β-estradiol for 24 and 48 hours, respectively, for estrogen regulated genes, using random 13-mer upstream primers and anchored poly-dT downstream primers. Application of more than 400 different primer combinations resulted in detection of almost 100 different estrogen-responsive genes. Some of these are listed

in Table 1 (see below), together with their corresponding accession numbers, upstream primer sequences, and the size and regulation of the bands

Table 1. Selected estrogen regulated genes in human breast cancer cells.

Identity	Accession No.	Upstream Gene Specific Primer	Downstream Primer	Size (bp)	Regulation
α1-antichymotrypsin	J05176	CCCTCCTTTCTGCATTAG[a]	HT11C	380	↑
EBP50	AF015926	AGCACTGATTCCCAGTTA	HT11GG	600	↑
p-Liv	U41060	GTGCTTCAGTGCT[a]	HT11GA	150	↑
PS2	X00474	TTCCTGGTGTCAC[a]	HT11G	350	↑
EST	AC005384	CAAAGGTACTCCTTTAT	HT11GG	250	↑
EST	AA677552	AGCACTGATTCCCAGTTA	HT11CT	180	↑
EST	AA291280	CACGCATAGACTG	HT11AC	170	↑
Monoamine oxidase A	X60819	CTGTCTGTCCCAGTTAA[a]	HT11GG	250	↓
Ribosomal Protein L9	U09953	ACTACCTCAGTTCTCAA	HT11CC	300	↓
TGFβ3	X14149	CACGCATAGACTG[a]	HT11AG	140	↓
EST	AA731207	AGCACTGATTCCCAGTTA	HT11CC	280	↓
EST	AA772139	AGCACTGATTCCCAGTTA	HT11CC	200	↓
EST	H81588	AGCACTGATTCCCAGTTA	HT11GG	300	↓

Arrows indicate regulation by 17β-estradiol: upregulated (↑), downregulated (↓).[a] indicates that the upstream primer is a targeted primer. Downstream primer sequences are presented in Table 3.

Abbreviations: EBP50, ezrin-radixin-moesin binding phospho protein; EST, Expressed Sequence Tag; TGFβ3, transforming growth factor β3.

Since most primer combinations result in the display of 100-150 bands, the expression levels of 40-60.000 bands have been investigated. Comparing this with the 20.000-30.000 genes that are expressed in a human cell at a given time, suggests that the majority of the estrogen regulated genes in MCF7 cells may have been detected.

Example 3

Identification of Estrogenic Chemicals.

Four marker genes were used to estimate the estrogenicity of the following compounds: 17β-estradiol (E2), zearalanone (ZA), diethylstilbestrol (DES), genestein (GS), nonylphenol (NP), bisphenol A (Bis-A), dibutylphthlate (DBP), methoxychlor (MC), endosulphan (ES), 1,1-dichloro-2,2-bis(p-chlorophenyl)ethylene (DDE), 4-hydroxytamoxifen (4-OH-TAM) and ICI 182.780 (ICI). The relative potencies of the tested compounds on the expression of the four genes are shown in Table 2, (seen below)

Table 2. Rank order of potencies of the estrogenic compounds on four marker genes.

Marker gene	**Rank order of potency**
PS2	E2 = DES > ZA > NP = GS > MC > ES = Bis-A > DDE > DBP
α1-ACT	E2 = DES > ZA > GS > NP > ES > Bis-A = MC > DDE > DBP
MAO-A	E2 > DES > ZA > NP > GS > DBP > Bis-A > ES = MC > DDE
TGFβ3	E2 = DES = ZA > NP = GS > Bis-A > MC > DDE > ES = DBP

Isolation of RNA.

Cells were harvested by addition of 1x trypsin-EDTA (Gibco BRL) and collected by centrifugation (1000 x g for 5 min.). Total RNA was prepared using the RNeasy total RNA kit (Qiagen) as described by the manufacturer, and stored in diethylpyrocarbonate treated H_2O (DEPC-H_2O) at -80°C. Contaminating DNA was removed from the total RNA by treatment with 5 u DNase-1 (Amersham/Pharmacia) in 20 mM Tris-HCl, pH 7.5 and 7.5 mM $MgCl_2$ at 37°C for 30 min. The DNase was removed by incubation with 25 µg/ml Proteinase K in 5 mM Tris-HCl, pH 7.5 and 10 mM EDTA, pH 8.0 at 37°C for 15 min before the RNA was extracted with phenol/chloroform and collected by ethanol precipitation. The total RNA was dissolved in DEPC-H_2O at a concentration of 1-5 µg/µl and stored at -80°C.

cDNA synthesis.

One µg total RNA and 0.5 µg HT11V primer (Table 3 as seen below) in 10 µl DEPC-H_2O were mixed and heated to 65°C for 1 min. The samples were quickly transferred to 42°C

where 10 µl cDNA Synthesis Mix and 7-8 U AMV Reverse Transcriptase (Stratagene) were added. The final composition of the reaction buffer was: 130 mM Tris-HCl, pH 8.3; 5 mM $MgCl_2$, 20 mM KCl and 0.625 mM each of dATP, dCTP, dGTP and dTTP. The samples were incubated at 42°C for one hour, before addition of 80 µl of 0.1 % Triton X-100 in H_2O. The samples were denatured at 95°C for 1 min and stored in aliquots at -80°C.

Table 3. Downstream Primers.

cDNA Synthesis & Differential Display (HT11V and HT11VN)

HT11A	AAGCTTTTTTTTTTTA
HT11C	AAGCTTTTTTTTTTTC
HT11G	AAGCTTTTTTTTTTTG

HT11AC	AAGCTTTTTTTTTTTAC
HT11AG	AAGCTTTTTTTTTTTAG
HT11GA	AAGCTTTTTTTTTTTGA
HT11GG	AAGCTTTTTTTTTTTGG
HT11CC	AAGCTTTTTTTTTTTCC
HT11CT	AAGCTTTTTTTTTTTCT

Amplification (T7HT11V)

T7HT11A	TAATACGACTCACTATAGGGAAGCTTTTTTTTTTA
T7HT11C	TAATACGACTCACTATAGGGAAGCTTTTTTTTTTC
T7HT11G	TAATACGACTCACTATAGGGAAGCTTTTTTTTTTG

Sequencing (T7)

SEQ-T7	**CY5**-TAATACGACTCACTATAGGGAA

Competitive PCR.

1 µl cDNA was used in competitive PCR reaction mixtures performed in total volumes of 12 µl of (final concentrations, including contributions from the cDNA): 12 mM Tris-HCl, pH 8.3; 50 mM KCl; 1.9 mM $MgCl_2$; 0.1 % Triton X-100; 0.005 % Gelatine; 14 µM each of dATP, dCTP, dGTP and dTTP; 1 µCi [^{35}S]αdATP (Amersham/Pharmacia); 10 pmol each of up- and downstream primers (Tables 1 and 3) and 1U AmpliTaq (Amersham/Pharmacia). PCR was performed in a Perkin-Elmer 9600 PCR machine and the cy-

cle conditions were: 1 cycle of 2 min at 95°C; 40 cycles of: 30 sec at 95°C, 1 min at 40°C, 1 min at 72°C and finally 1 cycle of 5 min at 72°C. After PCR, 10 µl loading buffer (8 % Ficoll 400, 10 mM EDTA; 10 mM NaOH; 0.1225 % Bromophenolblue; 0.1225 % Xylene cyanol in formamide) was added and the samples were denatured for 2 min at 96°C before they were loaded onto a 5 % polyacrylamide "sequencing type" gel run on the ALF-Express sequenator (Amersham Pharmacia Biotech). Electrophoresis was at 25W for 3 hours; after electrophoresis, gels were transferred to Whatmann 3MM paper, dried and analysed by autoradiography and phosphor imaging.

Amplification of cDNA fragments from differential display gels.

Differentially expressed bands were excised from the dried gels and the DNA content recovered by shaking the sample in 50 µl Te buffer (10 mM Tris-HCl, pH 7.5; 0.1 mM EDTA) at 95°C for 15 min. Five µl was used for PCR amplification in a total volume of 27 µl of (final concentrations): 10 mM Tris-HCl, pH 8.3; 50 mM KCl; 1.8 mM $MgCl_2$; 0.1 % Triton X-100; 0.005 % Gelatine; 70 µM each of dATP, dCTP, dGTP and dTTP; 2.5 U AmpliTaq (Perkin-Elmer), 10 pmol upstream primer and 10 pmol "extended" downstream primer (T7HT11V; Table 3). Cycle conditions were as described for differential display above, except that the annealing temperature was 42°C. Reamplified DDRT-PCR fragments were purified from 2% agarose gels as following: The fragments were carefully excised from the agarose gel and transferred to tubes containing small siliconised glass wool plugs. A hole was pinched in the bottom of each tube, and the DNA/buffer spun out of the agarose piece and into another tube.

Sequencing reactions were performed as cycle sequencing using the ThermoSequenase enzyme (Amersham Pharmacia Biotech) and a "T7 promotor complementary" primer (Seq-T7; Table 3) that matches all fragments amplified with the extended T7HT11V primers. All sequencing and DDRT-PCR gel electrophoresis was made on ALFexpress sequenators (Amersham Pharmacia Biotech).

Quantification of expression levels.

DDRT-PCR gels and Northern blots were analysed on a Fujifilm Bas-2500 phosphor imager. Exposure time was adjusted to the level of radioactivity on the gels/blots (between 3 and 24 h). Lane-to-lane variation in intensity was normalised either by counting a con-

stant band or by correcting according to the background immediately above or below the quantified band. The marker band in one lane was framed, using the software supplied with the phosphor imager, and the same frame was then copied to the corresponding band in the other lanes, to ensure that identical areas were used in all lanes. In order to normalise the intensity of the different lanes, the "marker band" frames were subsequently copied together and moved to another position, covering either a constant band or a blank area (background), close to the marker band. All frames were then counted by the phosphor imager software and the data was transferred to MS Excel. One lane was selected and the ratios between a constant band or background in that lane and the values counted at the corresponding position in each of the other lanes, were calculated. These ratios were used to normalise the intensities of the marker bands by multiplying the counts in each marker band, after subtracting the background value determined in that lane, with the corresponding ratio.

The following formula was used for normalisation (MB, Marker Band; BG, BackGround; CB, Constant Band): MB-level-lane-1 = (MB-lane-1-BG-lane-1)*CB-lane-1 / CB-lane-1; MB-level-lane-2 = (MB-lane-2-BG-lane-2)* CB-lane-1 / CB-lane-2; etc.

Optimisation of PCR.

For the 20 originally selected genes, PCR was improved by optimising the match between primer and mRNA, since alignment of the "random" upstream primer sequences used in the screening and the mRNA sequence in most cases revealed several mismatches. Targeted upstream primers were designed by correcting the mismatches and extending the primer with 4-5 extra nucleotides at the 5'end. Hence, combining the targeted upstream primer with the appropriate downstream primer results in a band of the same size as in the screening. Since the primer position is important for the efficiency of PCR amplification, several targeted primers were tested for each gene, and the primer that resulted in the strongest band selected for further testing.

In most cases, the use of targeted primers significantly increased the sensitivity of the assay. For example, with a "random" upstream primer, containing three mismatches within the 3'-six nucleotides of the primer, a concentration of 10^{-10}M 17β-estradiol was required to induce a detectable increase in the expression of pS2 (Fig. 9A) whereas application of a targeted upstream primer reduced the required concentration to only 10^{-12} M (Fig. 9B).

To further enhance the assay, it can sometimes be an advantage to reduce the number of competing bands. This can be done by replacing the one-base-anchored downstream primer (HT11V; Table 3) with a two-base-anchored primer (HT11VN; Table 3). In this study, two-base-anchored downstream primers for TGFβ3 and MAO-A were used.

Rapid induction of marker genes by 17β-estradiol.

The observed changes in gene expression could, in principle, be caused by the activation/repression of other genes, for example a transcription factor that is directly regulated by estrogen. The expression profiles of genes known to be regulated directly by estrogens suggest that primary effects of estrogen are induced within a few hours, whereas secondary effects are not detectable before 16-72 hours incubation. Therefore a time course study was performed where MCF7 cells were exposed to 17β-estradiol for increasing periods of time (0, 2, 4, 8, 16, 24 and 48 hours). For almost all estrogen-responsive genes detected in the screening, including the four marker genes presented in the present application, the changes in gene expression were detectable within 2-8 hours, suggesting that they are primary effects of 17β-estradiol (Fig. 10).

Estrogen- induced changes in marker gene expression are ER-mediated.

To verify that the regulation was mediated by the estrogen receptor(s) (ERs), the ability of the pure antiestrogen, ICI 182.780, to inhibit the 17β-estradiol induced changes in gene expression was tested. For all four marker genes, ICI 182.780 abolished the effect of 17β-estradiol in a dose-dependent manner (Fig. 11), demonstrating that the observed effects are mediated via the ERs. In fact, ICI was able to further reduce or induce the expression levels as compared to ethanol-only samples, which suggest that there are trace amounts of estrogens in the stripped serum, whose effects are reversed by ICI. The antiestrogen-mediated repression of α1-ACT is difficult to detect because the concentration of 17β-estradiol used in the competition experiment was relatively low (10^{-11} M) and a 10-fold higher concentration is required to significantly induce α1-ACT expression (see below).

The results presented herein are most likely only derived from activation of ERα, since the inventors have not been able to detect the ERβ mRNA in MCF7 cells by RT-PCR, not even with nested primers and 2 X 40 PCR cycles.

Consistency of the technology.

It has already been shown that MCF7 cells respond strongly to estrogens, however, it is important that the technology used to assay gene expression is sufficiently robust and results in reproducible results. Since the expression levels are determined by competitive PCR amplification of cDNA synthesised from different RNA samples, it is important that the protocols do not introduce significant variation. In addition, since it is impossible to obtain completely identical loading on DDRT-PCR gels, it must be possible to normalise the lanes, either according to the background or a constant band.

To test the consistency of the method, 3 cDNA synthesis reactions were performed from a single RNA preparation, followed by 3 PCR of each cDNA, and the results for the pS2 primer set are shown in Fig. 12A. The DDRT-PCR gel was scanned by phosphor imaging and the results normalised according to the background just below the pS2 band (Fig. 12B). This showed that the variation introduced by the cDNA synthesis and PCR was less than 24% and 16%, respectively, and that the maximal variation between two samples was less than 34%. These differences are representative for the variation that have been observed among the hundreds of samples that have been analysed and this variation is small compared with the several fold differences among samples treated with different concentrations of estrogens.

To test the power of the normalisation, 2/3 and 1/3 of the volume of the sample in lane 9 (sample C/3) were loaded separately (Fig. 12A, Lanes 10, 11 and 12). After normalisation, the pS2 values in these lanes were essentially identical, showing that normalisation according to the background or a constant band is capable of normalising samples, even when the loaded amounts differ with 300% (Fig. 12B, Lanes 10, 11 and 12).

Optimisation of the endogenous gene Expression assay.

The most important parameter when selecting a marker gene is, that its expression is highly sensitive to the treatment, i. e. the intensity of the corresponding band must be very different in estrogen treated and untreated cells. Moreover, the gene must be directly regulated by estrogens, and not be dependent on prior induction of another gene, and, finally, its induction must be reversed by the pure anti-estrogen ICI 182.780.

To test the different candidate marker genes (see below), MCF-7 cells were exposed to either increasing concentrations of test compound for 24 hours or a fixed concentration for

different time periods. The candidate genes were then assayed for dose- and time-dependent effects on mRNA expression as reflected in the intensity of the corresponding bands. The initial selection reduced the number of candidate marker genes to about 20 that all responded strongly to estradiol.

As an example, but not meant to be limiting for the present invention, estrogen-dependent expression profiles are presented for four representative marker genes: pS2, monoamine oxidase A (MAO-A), transforming growth factor β3 (TGFβ3) and α1-anticymotrypsin (α1-ACT).

Example 4

Induction of pS2 mRNA expression.

Induction of the pS2 mRNA is a widely used indicator of estrogenicity (Soto et al., 1995; Zava et al., 1997; Jakowlew et al., 1984), and the pS2 mRNA was detected several times in the screening. It is rapidly and strongly induced by estrogens and was therefore selected as one of the marker genes. Since its expression is so strongly correlated to the level of estrogen, it is routinely, as a first approach, tested if a compound has the ability to induce the pS2 gene. Hence, for all test compounds, induction of pS2 mRNA was assayed by DDRT-PCR (Fig. 13) and the expression profiles quantified by Phosphor Imaging (Fig. 14).

The expression level of pS2 mRNA was increased in a dose-dependent manner by all the tested estrogens, however, their potencies were very different. Based on their relative potency (Table 2), the chemicals can be divided into 3 groups. Group 1 include E2, ZA and DES, which cause a detectable increase in pS2 at 10^{-12}M to 10^{-11}M, Group 2 include NP and GS, which induce pS2 at 10^{3}-10^{4} fold higher concentrations (>10^{-8}M) and Group 3 include BisA, DDE, MC, ES and DBP, that require 10^{5}-10^{6} fold higher concentrations, relative to E2, to induce pS2 (Figs. 7 and 8). The high-potency estrogens in Group 1 (E2, ZA and DES) cause a significantly greater quantitative change in the expression level of pS2, compared to the environmental estrogens in Group 2 and 3. For example, the expression level of pS2 is increased almost 25-fold after exposure to E2 (10^{-10} M), but less than 10 fold after exposure to DBP (10^{-4} M) (Figs. 7 and 8).

A slight increase in the expression level of pS2 mRNA was also observed after exposure to high concentrations (>10^{-7}M) of 4-OH-TAM (Figs. 7 and 8).

Example 5

Dose-dependent effects of estrogens and anti-estrogens on three other marker genes.

To increase the strenght of the estrogenicity assay, and to analyse whether other genes responded as pS2, an assya was performed on dose-dependent effects of the test compounds on the three other marker genes.

Exposure to increasing concentrations of E2 caused an induction of α1-ACT, and a reduction in the expression levels of TGFβ3 and MAO-A (Fig. 5). Detectable reductions in the expression levels of TGFβ3 and MAO-A could be detected already at an E2 concentration of 10^{-13}M (Fig. 5), equivalent to, or even below, the dose required to induce pS2 mRNA expression (Figs. 13 and 14) whereas detectable induction of α1-ACT required a 100-fold higher concentration (10^{-11}M) (Fig. 5). The other test compounds caused detectable changes in the expression levels of TGFβ3 and MAO-A, respectively, at doses similar to those required for pS2 induction, whereas, for most chemicals, the concentrations required to induce α1-ACT were 10-100 fold higher (Fig. 5).

There are significant marker-gene-dependent differences in the rank order of potencies of the test compounds (Table 2). For example, the potencies of E2, DES and ZA were essentially identical on TGFβ3, whereas the potency of ZA was almost 100-fold lower than the potency of E2 and DES on pS2, α1-ACT and MAO-A. Furthermore, NP and GS affected the expression levels of pS2 and TGFβ3 with similar potencies, whereas the potency of NP was almost 10-fold higher than the potency of GS on MAO-A, whereas the potency of NP on α1-ACT was lower than that of GS (Table 2).

The partial estrogen agonist, 4-OH-TAM, resulted in slightly increased expression levels of α1-ACT and pS2, respectively, and a reduction in the expression level of MAO-A, suggesting that 4-OH-TAM acts as a partial agonist on these genes. In contrast, a high concentration of 4-OH-TAM resulted in a slightly increased expression of TGFβ3 (Fig. 5), suggesting that it acts as an antagonist or a Selective Estrogen Receptor Modulator (SERM) on TGFβ3.

Example 6

Comparisson of the estrogenic potency of the synthetic estrogen Zeranol and five related compounds

To compare the estrogenic potency of Zeranol to the high-potency estrogens 17β-estradiol and DES and to a phytoestrogen (genistein) and a putative endocrine disrupter (Bisphenol A) (Fig. 1), human MCF7 cells were exposed to a range of concentrations of the compounds. The expression levels of six endogenous genes were subsequently analysed by a competitive PCR method, and the PCR products were visualised by polyacrylamide gel electrophoresis, similar to the Differential Display method (Liang & Pardee, 1992), as described above. The resulting bands were then quantified by phosphor imaging.

Cell culturing and hormone exposure

All cell cultures were made in 25cm^2 flasks. Samples with ethanol, ICI 182.780, 17β-estradiol, DES, Zeranol, genistein and Bisphenol A were prepared in four independent replicas and samples with Zeranol metabolites were prepared in duplicates. Human estradiol-dependent MCF-7 breast cancer cells were grown in a DMEM medium (Gibco BRL) containing 5 % foetal bovine serum (FBS) (Gibco BRL), 1 nM insulin (Boehringer Mannheim), 2 mM L-glutamine (Gibco BRL), 1x non essential amino acids (Gibco BRL) and 25 IU/ml Penicillin-Streptomycin (Gibco BRL). Six days before the cells were used for testing chemicals, the medium was substituted with a DMEM medium without phenol red (Gibco BRL), containing 5 % dextran-charcoal stripped FBS, and otherwise as the standard medium, as previously described. Testing was made on 40-80% confluent cells.

Test chemicals

The test compounds were: Zeranol (α-zearalanol) (Sigma, Z-0292); 17β-estradiol (Sigma, E-2758); diethylstilbestrol (DES) (Sigma, D-4628); Bisphenol A (Sigma, I-0635); and genistein (Sigma, G-6649). Samples treated with 100 nM ICI 182.780 (ICI) (Zeneca Pharmaceuticals) were included as an anti-estrogen controls in all experiments. α-zearalanol and its metabolites (α-zearalenol; β-zearalanol; β-zearalenol; zearalenone; and zearalanone) were obtained both from Sigma (Z0292, Z0166, Z0417, Z2000, Z2125,

Z0167, respectively) and from The European Reference Laboratory, Laboratory for Residue Analysis, NL 3720 BA Bilthoven, The Netherlands (See figure 15).
The tested concentrations depended on the potencies of the compounds, which were determined in pilot experiments. For 17β-estradiol, DES and Zeranol the range was from 10^{-15}M (1 fM) to 10^{-11}M (10 pM), for ATB0+ 10^{-10}M (100 pM) Zeranol was also included. For genistein the range was from 10^{-10}M (0.1 nM) to 10^{-5}M (10 µM) and for Bisphenol A the range was from 10^{-9}M (1 nM) to 10^{-5}M (10 µM).

Isolation of RNA, cDNA synthesis, competitive PCR and amplification of cDNA fragments from differential display gels

Cells were harvested and the RNA was extracted as described above. Sequencing reactions were performed as cycle sequencing using the ThermoSequenase enzyme (Amersham Pharmacia Biotech). All sequencing and DDRT-PCR gel electrophoresis were run on ALFexpress sequenators (Amersham Pharmacia Biotech). Detailed step-by-step manuals for all procedures related to DDRT-PCR can be obtained from the web site: http://www.biobase.dk/~ddbase/DD-Manuals.html

Quantification of expression levels and data analysis

DDRT-PCR gels were analysed on a STORM 820 phosphor imager (Amersham-Pharmacia-Biotech). Exposure time was adjusted to the level of radioactivity on the gels (6 - 24 h). Lane-to-lane variation in intensity was normalised according to either the background above or below the quantitated band or a constant band. Following formula for normalisation was used:

MB-level-lane-1 = (MB-lane-1 - BG-lane-1)*CB-lane-1 / CB-lane-1;
MB-level-lane-2 = (MB-lane-2 - BG-lane-2)* CB-lane-1 / CB-lane-2; etc.
(MB, Marker Band; BG, Back Ground; CB, Constant Band).

If the background was used for normalisation, BG replaces CB. The choice of normalisation parameter (background or constant band) did not affect the results. All values were then calculated as fold induction or reduction compared to the average of the four (or two) samples from cells treated with 100 nM of the anti-estrogen ICI 182.780. The average and the standard deviation were then calculated and all data points that are derived from four independent samples (due to occasional bad lanes a few of the results

are only based on three samples) are represented as average ± standard deviation. For results obtained from analysis of duplicate samples (Zeranol metabolites), the values represent the average of the two samples. The results (raw data) based on four samples (for a few only three) were evaluated statistically by Student's t-Test, assuming two-tailed distribution and two-samples equal variance.

Induction of PS2 and ATB0+

The PS2 mRNA was displayed with a primer combination as described in table 4.

Table 4: Primer combinations

	upstream primer	donstream primer
PS2	TTCCTGGTGTCAC	AAGCTTTTTTTTTTG
ATBO+	TTCAGAGTCCACA	AAGCTTTTTTTTTTTGA
TGFβ3	CACGCATAGACTG	AAGCTTTTTTTTTTTAG
Monoamine Oxidase A	CTGTCTGTCCCAGTTAA	AAGCTTTTTTTTTTTGG
MRG1/p35srj	TCTCAGTGCTTCAGTGCT	AAGCTTTTTTTTTTTGG
GST mu3	CCTATGATATCTTGGAT	AAGCTTTTTTTTTTTGA

A significant induction could be detected at a concentration of about 1 pM of 17β-estradiol, DES and Zeranol, whereas almost 10 nM genistein and 100 nM Bisphenol A were required for a similar induction (Fig. 16A; Table 5). A slight induction of PS2 could, in some experiments, be detected at 0.1 pM of 17β-estradiol, DES and Zeranol (not shown). The PS2 mRNA can be induced almost 200 fold by high concentrations of many estrogens and the apparently smaller induction with the high-potency estrogens, as compared with genistein (Fig. 16A), was caused by the chosen concentrations; higher concentrations of 17β-estradiol, DES and Zeranol (and genistein and Bisphenol A) led to higher induction. The maximum induction of the PS2 mRNA by different estrogens varied, but high concentrations of the three high-potency estrogens always led to larger induction of PS2 than high concentrations of medium- and low-potency estrogens (such as Genistein, Bisphenol A and DBP) (Jørgensen *et al.*, 2000; results not shown). "Fold

induction" is very sensitive to differences in the level in ICI 182.780 treated cells, which always is low, but a litle variable, leading to different "fold induction" despite essentially similar expression levels, in treated samples.

Table 6: Relative potencies of 17β-estradiol, DES, Zeranol, Genistein and Bisphenol A

	17β-estradiol	DES	Zeranol	Genistein	Bisphenol A
PS2	IIIII	IIIII/ IIII	IIII	II	I
ATBO+	IIIII	IIII	III	II	I
TGFβ3	IIIII	IIIII	IIIII	II	I
Monoamine Oxidase A	IIIII	III	III/ IIII	II	I
MRG1/p35srj	III	IIII	IIIII	II	I
GST mu3	IIIII	II	III		

ATB0+ encodes Amino acid Transporter B0+ (ATB0+), a membrane protein with twelve transmembrane domains that is related to many sodium- and chloride-dependent amino acid transporters (Sloan & Mager, 1999). In MCF7 cells, the ATB0+ mRNA was induced up to 6 fold by moderate (relative to each compound) concentrations of all the estrogens we have tested, but contrary to the PS2 mRNA, ATB0+ was not further induced by higher doses (Fig. 16B). Thus, the maximum induction, corresponding to 5-6 fold, was similar for all the tested high-, medium- and low-potency estrogens. The relative potencies of the high potency compounds were different from that determined from PS2 (Table 5), with one order of magnitude difference between (in decreasing potency) 17β-estradiol, DES and Zeranol whereas the relative potencies of Genistein and Bisphenol A was similar to the results from PS2 (Table 5).

Estrogenicity determined from monoamine oxidase A and TGFβ3

To further elucidate differences in potency on different genes, the expression levels of two other estrogen-regulated genes were analysed monoamine oxidase A and TGFβ3. Both showed minor differences compared to PS2 and ATB0+ (Table 5). For monoamine oxidase A the potency of 17β-estradiol was greater than that of DES, and Zeranol was slightly less estrogenic than DES. For TGFβ3, the potency of 17β-estradiol, DES and Zeranol was essentially identical. The expression of TGFβ3 was less sensitive to Genistein and Bisphenol A than the other four genes; a down regulation could be detected with 0.01 - 0.1 µM genistein and 0.1 - 1 µM Bisphenol A 1 (Table 5).

GST mu3 and MRG1 / p35srj are extremely sensitive to estrogens

A few genes responded to very low concentrations of the high potency estrogens. One encoded glutathione S-tranferase (GST) mu3, a member of the phase-two detoxification enzymes that protect the cell, and especially the DNA, against damage from free oxygen radicals (Hayes & McLellan, 1999). GST mu3 was down regulated by all the estrogens tested and was especially sensitive to the three high potency compounds (Fig. 17A; Table 5). GST mu3 was sometimes significantly down regulated by ICI 182.780, and the level in ICI 182.780 treated samples was always lower that in the ethanol treated sample (Fig. 17A), which therefore was used for the statistical evaluation. Down regulation could be detected already at 1 - 10 fM concentrations of 17β-estradiol whereas a concentration of about 1 pM of Zeranol and almost 10 pM DES was required for a similar down regulation (Fig. 17A). For medium and low potency estrogens the results were less consistent, with relatively large differences between results obtained with GST mu3 and other genes (not shown). Furthermore, almost all other estrogen regulated genes assayed responded to estrogens within 2-8 hours (as shown above) whereas a down regulation of GST mu3 required about 16 hours of treatment.

MRG1 / p35srj was first reported as a cDNA that was related to a melanocyte specific cDNA and it was named Melanocyte-specific-gene-Related-Gene 1 (MRG1) (Shioda *et al.*, 1996), later it was shown that MRG1, now called p35srj, was a p300/CBP binding protein, involved in regulation of the activity of p300/CBP (Bhattacharya *et al.*, 1999). MRG1 / p35srj was down regulated by all the estrogens tested, and it was especially sensitive to Zeranol, since a down regulation could be detected at a concentration of about 10 fM whereas 17β-estradiol was at least three orders of magnitude less potent and the potency of DES was in between Zeranol and 17β-estradiol (Fig. 17B; Table 5). The

timing of the down regulation was similar to the majority of the estrogen-regulated genes, and could be detected after 4-8 hours of treatment.

Estrogenicity of Zeranol metabolites

To investigate the estrogenic potencies of Zeranol metabolites, MCF7 cells were exposed to increasing concentrations of six different compounds: α-zearalanol, α-zearalenol, β-zearalanol, β-zearalenol, zearalenone, and zearalanone (Fig. 18). All samples were prepared in duplicates, using compounds from both Sigma and from The European Reference Laboratory, the potencies of compounds from Sigma and from The European Reference Laboratory were identical. The expression levels of the six genes described above were subsequently analysed (Table 6) and the results from analysis of ATB0+ are shown in Fig. 18.

The relative potencies of the six compounds were essentially identical for all six genes (Table 6). Zeranol (α-zearalanol) and α-zearalenol had similar potencies and both were significantly more estrogenic than the other compounds. PS2, TGFβ3 and monoamine oxidase A were more sensitive to α-zearalenol than to Zeranol (α-zearalanol) whereas the opposite was observed for ATB0+; MRG1 was equally sensitive to the two compounds. The rank order for the remaining four compounds was similar for the five genes, although there were minor differences (Table 6). Note that the mycoestrogen zearalanone was several orders of magnitude less estrogenic than the synthetic estrogen Zeranol (α-zearalanol).

Table 6: Relative potencies of Zeranol metabolites

	α-zearalenol	Zeranol	zearala-none	zearale-none	β-zearalanol	β-zearale-none
PS2	IIIIII	IIIII	IIII	III	II	I
ATBO+	IIIII	IIIIII/ IIIII	IIII/ III	III	III	II
TGFβ3	IIIIII	IIIII/ IIII	IIII	III	II	I
Monoamine Oxidase A	IIIIII	IIIII	IIII	III	II	II
MRG1/p35 srj	IIIIII	IIIIII	IIIII	IIII	III	II

Figure legends.

Figure 1.

Chemical structures of the compounds used in the study.

Figure 2.

Screening for estrogen-regulated genes in MCF7 cells. MCF7 cells were cultivated for 6 days in estrogen-free media before they were transferred to media containing the test compounds for 24h. All samples were made in dublicates. The test compounds were: ethanol (vehicle); E2, 17β-estradiol (10 nM); DES, diethylstilbestrol (synthetic estrogen) (10 nM); ZA, α-zearalanol (synthetic estrogen, used for growth promotion in catle) (10 nM); GS, genistein (phytoestrogen) (10 µM); ES, endosulphan (pesticide) (10 µM); MC, methoxyclor (pesticide) (10 µM); NP, nonylphenol (surfactant) (10 µM); ICI, ICI182.780 (steroid anti-estrogen) (100 nM). The endogenous marker gene was Transforming Growth Factor β3 (TGFβ3), and the primers were, upstream CACGCATAGACTG, downstream AAGCTTTTTTTTTTTAG. TGFβ3, represented by a double band, is downregulated by all the estrogens, whereas its expression is unaffected by ICI 182.780.

Figure 3.

Gene expression during embryogenesis. This is an example of how gene expression during embryogenesis can be analysed by DDRT-PCR. The RNA samples were derived from testes, disected from foetuses, pups and adult mice. The primers were: upstream: GTATTTGCAGGGC; downstream AAGCTTTTTTTTTTG. PC and PN denotes post coitus and post natal, respectively. The identity of six bands that have been identified by direct sequencing is indicated, with their corresponding sequence accession numbers and phosphor imaging quantification of these bands is shown in the boxes. The intensity of the lanes was normalised according to the background in each lane before quantification. Note that the expression of a very high percentage of the displayed mRNAs changes during the 65 days of development covered by the samples. The image corresponds to one half of the DDRT-PCR gel. The other half included similar samples, but from DES treated mice, however, none of the bands displayed by this primer combination were affected by DES treatment. The appearance of the band corresponding to the sperm tail structural protein odf2 correlates with,

or maybe slightly precedes, the onset of spermatogenesis (it appears at day 14). Several other bands show a similar regulation (indicated by arrow heads), suggesting that their expression is also related to spermatogenesis.

Figure 4.

The response to estrogens is highly cell-type-specific. Human breast cancer MCF7 and T47D cells were grown in estrogen-free media, containing charcoal stripped foetal calf serum, for 6 days (- Estradiol) before some were transferred to media containing 1 nM 17β-estradiol for 24h (+ Estradiol). The primers that were used to display the pS2 gene were: upstream, TTCCTGGTGTCAC; downstream, AAGCTTTTTTTTTTTG. The pS2 gene is one of the most sensitive markers for estrogens in MCF7 cells, however, it is apparently not expressed in T47D cells, and hence not regulated by estrogens in this cell line. Other bands, each corresponding to a specific mRNA, that are conserved between the two cell lines are indicated by arrow heads. The Differential Display technology (Liang & Pardee, 1992).

Figure 5.

Phosphor imaging estimates of the dose-dependent effects of estrogens on the expression level of A) α1-ACT; B) MAO-A and C) TGFβ3. MCF7 cells were exposed to increasing concentrations of the test compounds, and harvested after 24 hours. Fold induction / repression is relative to EtOH and the molar concentrations are indicated. Primers were as in Fig. 10. Bands corresponding to each of the three marker genes were displayed by DDRT-PCR, and the expression profiles were quantitated by phosphor imaging. An example of a DDRT-PCR expression profile (corresponding to E2-exposed cells), is inserted in each chart. The results are representative of at least 2-3 independent experiments.

Figure 6.

Endogenous gene expression as a marker for estrogenicity. The estrogenicity of DES was tested using an endogenous gene (TGFβ3). MCF7 cells, exposure, primers and method were as in Fig. 2, except that a dose-response experiments was performed. Cells were exposed to increasing concentrations of DES and the expression level of TGFb3 was subsequently determined in each sample. A, Differential Display gel; B, phosphor imaging quantification of the bands corresponding the TGFβ3; the lanes were normalised according

to the background before analysis. Note that the expression level of TGFβ3 correlates completely with the DES concentrations in the culture media.

Figure 7.

Expression of putative estrogen-regulated endogenous genes in blood. MCF7 cells were treated as in Fig. 2 & 6, and a band corresponding to the monoamine oxidase A mRNA displayed by a primer combination (upstream, CTGTCTGTCCCAGTTAA; downstream AAGCTTTTTTTTTTTGG). A, Differential Display gel,; B, quantification of the monoamine oxidase A band. The 17β-estradiol concentrations in the cell culture media (MCF7) is shown together with the measured (by RIA) 17β-estradiol concentrations in the individual blood samples. The red blood cells were lysed before RNA purification, and the results are thus only derived from analysis of white blood cells. The monoamine oxidase A gene is down-regulated by estrogens in MCF7 cells. However, although it is expressed in cells of the blood, its expression level in blood does not correlate with the serum concentration of 17β-estradiol. However, we have not corrected for SHBG binding, and the "real" concentrations of free 17β-estradiol are thus unknown.

Figure 8.

Adjustment of gene expression to estrogen-free cell culture conditions. MCF7 cells, cultured in a standard DMEM medium (DMEM), were transferred to an estrogen free medium and cells were harvested on 6 consecutive days. The band corresponding to pS2 (indicated by an arrow) was displayed by the DDRT-PCR technique (A) and a quantitative estimate of the relative change in gene expression was obtained by Phosphor Imaging (B). The primers were: pS2 and HT11G (Tables 1 and 2).

Figure 9.

Application of targeted upstream primers increases the sensitivity. Cells were exposed to ethanol (control) or increasing concentrations of E2 (10^{-13}M - 10^{-9}M). The band corresponding to the pS2 mRNA was displayed by DDRT-PCR using either a non-targeted upstream primer (p-Liv) and HT11G (A) or a targeted upstream primer (pS2) and HT11G (B) (Tables 1 and 2). The positions of the bands corresponding to pS2 are indicated by arrows.

Figure 10.

Time-dependent effects of 17β-estradiol on marker gene expression. MCF7 cells were exposed to 10^{-8}M E2 for 2, 8, 16, 24 and 48 hours. Control cells received ethanol (EtOH) for 24 hours. Bands corresponding to the four marker genes (pS2, α1-ACT, TGFβ3 and MAO-A) were displayed by DDRT-PCR, and the positions of the bands are indicated by arrows. The primers were: pS2/HT11G (pS2); α1-antichymotrypsin/HT11C (α1-ACT); TGFβ3/HT11AG (TGFβ3) and monoamine oxidase A/HT11GG (MAO-A) (Tables 1 and 2).

Figure 11.

Dose-dependent inhibition of E2-mediated changes in gene expression by ICI 182.780. MCF7 cells were exposed to a fixed concentration of E2 (10^{-8}M) and increasing concentrations of ICI 182.780 (10^{-12}M - 10^{-6}M) for 24 hours. Bands corresponding to the marker genes were displayed by DDRT-PCR, and the positions of the bands are indicated by arrows. The primers were as in Fig. 10.

Figure 12.

Competitive PCR amplification is a highly consistent technology. From a single RNA sample, three independent cDNA synthesis reactions were performed (A, B and C), followed by three PCR reactions of each cDNA (1, 2 and 3). The band corresponding to pS2 was displayed by DDRT-PCR (A), using the same primers as in Fig. 8b. Equal volume of the sample in lane 9 (C/3), was loaded in lane 10 (vol=1), and 2/3 and 1/3 the volume in lane 11 (vol=2/3) and 12 (vol= 1/3), respectively. After quantification by phosphor imaging, the values were normalised acdording to the background in each lane and the result is shown in (B).

Figure 13.

Dose-dependent induction of pS2 in MCF-7 cells by different estrogens. MCF7 cells were exposed for 24 hours to E2, DES, ZA, GS, NP, Bis-A, MC, DDE, DBP, ES, 4-OH-TAM and ICI. Control cells received ethanol (EtOH) or ICI (10^{-7}M) for 24 hours. The band corresponding to pS2 (indicated by arrows) was displayed by DDRT-PCR. The primers were as in Fig. 8b and quantification of the expression levels are shown in Fig. 14.

Figure 14.

Quantitative representation of DDRT-PCR expression profiles. Fold induction is relative to EtOH and the molar concentrations are indicated. To eliminate lane-to-lane variations, the quantitations were normalised according to the background in each lane. The results are representative of at least 2-3 independent experiments.

Fig. 15.

Zeranol (α-zearalanol) and related compounds. All are beta resorcylic acid lactones and each can be metabolised / converted into all the other compounds, albeit with different efficiencies (Thouvenot *et al.*, 1981; Migdalof *et al.*, 1983).

Figure 16.

Relative potencies determined from analysis of PS2 and ATB0+ expression levels. (A), induction of PS2; (B), induction of ATB0+. MCF7 cells were incubated with the indicated concentrations of the different compounds (indicated at the bottom). Each column represents the average of four independent cell cultures and the error bars indicate the standard deviation. Values (left) indicate fold induction compared to samples treated with 100 nM of the anti-estrogen ICI 182.780. The significance (Student's t-test) of the differences between the expression levels observed in treated samples compared to samples treated with ICI 182-780 is indicated: *, $P < 0.01$; **, $P < 0.001$; Significance is only indicated for the first sample (lowest concentration) that showed a significant difference. All samples treated with higher concentrations were also significantly different from the ICI 182.780 sample.

The apparently higher induction of PS2 by genistein (up to 60 fold) as compared to the about 30 fold for the high potency compounds, is caused by the high concentration of genistein; estradiol, DES and Zeranol all lead to more than 100 fold induction at 100 pM, similar induction (110 fold) was observed for 10 µM genistein (not shown). ATB0+ was induced by all the tested estrogens, however, contrary to PS2, ATB0+ could only be induced 5-6 fold, higher concentrations than those shown, did not lead to additional induction.

Fig 17.

GST mu3 and MRG1/p35srj are highly sensitive to the high-potency estrogens. GST mu3 (A) and MRG1/p35srj (B) are the most estrogen-sensitive genes we have detected.

Contrary to other estrogen regulated genes where ICI 182.780 treatment resulted in a slight reversal of the estrogen effect, GST mu3 was generally slightly down regulated by ICI 182.780 and therefore the ethanol-treated samples were used for the statistical evaluation (*, $P < 0.01$; #, $P < 0.005$; **, $P < 0.001$). For MRG1 / p35srj the ICI 182.780 samples were used for the statistical evaluation (*, $P < 0.01$; **, $P < 0.001$).

Figure 18.

Induction of ATB0+ by Zeranol and its metabolites. (A), Phosphor imager scan of a polyacrylamide gel. Each lane corresponds to analysis of a cell culture incubated with the indicated concentration of the compound. Only one sample at each concentration was loaded onto this gel. (B), quantification of the expression levels by phosphor imaging, each column represents a quantification of the corresponding lane in (A). Values to the left are the original readings from the STORM phosphor imager. The quantitations were normalised according to the back ground just below the ATB0+ band. Normalisation with a constant band (marked with n), instead of the back ground, resulted in identical quantitations (not shown). (C), summary of the expression results shown in (A) and (B) but based on two independent samples at each concentration. The values to the left indicate fold induction compared to the average of the two ICI 182.780 samples.

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Claims

1. A method for evaluating a cellular response to an environmental compound comprising determining or comparing the expression levels of at least one endogenous gene.

2. A method for evaluating a cellular response to an environmental compound comprising comparing the effect of said environmental compound on endogenous gene expression in a sample to the effect on endogenous gene expression of another compound to said sample.

3. A method for evaluating a cellular response to an environmental compound comprising comparing the effect of said environmental compound on endogenous gene expression in a sample to the known effect on endogenous gene expression of another substance to said sample.

4. A method according to any of claims 1-3, wherein said samples have prior been treated and/or exposed or have been suspected of being treated or exposed to an environmental compound of interest.

5. A method according to claim 4, wherein the environmental compound of interest is selected from the group consisting of any component of food, any component of cosmetics, any component of hygiene products, any component of pharmaceuticals and any component of smoke.

6. A method according to claim 4 or 5, wherein the environmental compound of interest is a chemical that interacts with an endocrine system.

7. A method according to claim 4 or 5, wherein the environmental compound of interest is a compound that interacts with an estrogen receptor.

8. A method according to claim 7, wherein the environmental compound of interest is a compound that interacts with ERβ and/or ERα.

9. A method according to claim 6, wherein the environmental compound of interest is a chemical that interacts with an orphan receptor.

10. A method according to claim 6, wherein the environmental compound of interest is a compound that interacts with an androgen receptor.

11. A method according to claim 6, wherein the environmental compound of interest is a compound that interacts with a thyroid receptor.

12. A method according to claim 6, wherein the environmental compound of interest is a compound that interacts with an aromatic hydrocarbon receptor.

13. A method according to claim 6, wherein the environmental compound of interest is selected from the group consisting of polychlorinated biphenyls (PCBs), organochlorine pesticides, alkylphenols, phthalates, food antioxidants, phytoestrogens and mycoestrogens.

14. A method according to any of the preceding claims, wherein the environmental compound of interest is a compound that is known or suspected to cause a disease.

15. A method according any of the preceding claims, wherein the environmental compound of interest is a compound that is known or suspected to cause cancer.

16. A method according to any of the preceding claims, wherein the endogenous gene is selected from a group of genes whose level of expression is sensitive to treatment with any of the compounds named in any of claims 5-15.

17. A method according to claim 16, wherein the endogenous gene is selected from a group of genes that are directly regulated by any of the compounds named in any of claims 5-15.

18. A method according to any of claim 16 or 17, wherein the endogenous gene is selected from a group of genes whose induction or repression of expression is reversed by treatment with anti-compounds to any of the compounds named in any of claims 5-15.

19. A method according to claim 18, wherein said gene is selected from the group of genes consisting of estrogen-responsive genes comprising pS2, monoamine oxidase A (MAO-A), transforming growth factor β3 (TGFβ3), α 1-anticymotrypsin (α 1-ACT), Alpha-tropomyosin, Prothymosin alpha, Calnexin, Plasma membrane Ca2+ ATPase, Alpha-1 antichymotrypsin and Nrf2 (NF-E2-like basic leucine zipper), Estrogen receptor alpha, c-myb, Rap1a GEF, GSTM3 (glutathione transferase mu 3), GSTM4 (glutathione transferase mu 4), Transforming growth factor beta3 (TGF beta3), Monoamine oxidase A, NAD(P)H:menadione oxidoreductase, NADH-ubiquinone oxidoreductase B8, Ezrin-radixin-moesin binding phospho protein, 14-3-3 beta.

20. A method according to any of the preceding claims, wherein the sample is selected from the group consisting of cell lines, blood samples, urine samples, organ samples and tissue samples of a mammal.

21. A method according to claim 20, wherein the cell-line is a hormone dependent cell system.

22. A method according to any of claims 20 and 21, wherein the mammal is a human.

23. A method according to any of claims 1-19, wherein the sample is selected from the group consisting of cell lines, blood samples, urine samples, organ samples and tissue samples of a fish.

24. A method according to any of claims 2-23, wherein said other substance is a hormone.

25. A method according to claims 24, wherein said other substance is selected from the group consisting of estrogens, androgens, thyroids, stress hormones and other endocrine active compounds.

26. A method according to claim 25, wherein said other substance is an estrogen.

27. A method according to any of claims 2-23, wherein said other substance is a carcinogen.

28. A method according to any of the preceding claims, comprising:

a) subjecting mRNA derived from the sample to reverse transcription and/or competitive PCR,

b) synthesising second strand cDNA complementary to the first strand DNA fragments and subjecting the obtained cDNA fragments to a molecular amplification procedure, wherein at least one of the provided deoxynucleotides or at least one primer is labelled,

c) separating the amplified cDNA fragments of step b) and assaying expression levels of mRNA by the amount of label incorporated during PCR.

29. A method according to any of the preceding claims, comprising:

a) subjecting mRNA derived from the sample to reverse transcription and/or competitive PCR, using at least one primer corresopnding to the general formula

$$AAGCT_{11}X_nY_m$$

wherein X is selected form the group consisting of deoxyadenosine (dA), deoxycitidine (dC) and deoxyguanosine (dG), Y is selected from the group consisting of deoxyadenosine (dA), deoxythymidine (dT), deoxycitidine (dC) and deoxyguanosine (dG), n=1 and m is an integer between 0 and 1,

b) synthesising second strand cDNA complementary to the first strand DNA fragments and subjecting the obtained cDNA fragments to a molecular amplification procedure, wherein at least one of the provided deoxynucleotides or at least one primer is labelled,

c) separating the amplified cDNA fragments of step b) and assaying expression levels of mRNA by the amount of label incorporated during PCR.

30. A method according to any of claims 28 or 29, wherein the incorporated label is selected from the group consisting of radioactive isotopes, fluorescent groups and biotin.

31. A method according to any of claims 28-30, wherein the assaying of expression level in step c) is quantitative.

32. A method according to any of claims 1-27, comprising:

a) immobilising different cDNAs on a solid support, and

b) comparing the expression of corresponding genes in different samples by hybridising labelled cDNA from each sample to said solid support.

33. Use of a method according to any of the preceding claims 1-32 in a toxicological analysis.

34. Use of a method according to any of claims 1-32 for diagnostics.

35. Use of a method according to any of claims 1-32 for diagnosing cancer.

36. Use of a method according to any of claims 1-32 for diagnosing asthma and/or allergy.

37. Use of a method according to any of claims 1-32 for evaluating the efficiency of a treatment for hormonal deficiency in a mammal in need thereof.

38. Use of a method according to any of claims 1-32 for evaluating the efficiency of a hormonal replacement therapy in a mammal in need thereof.

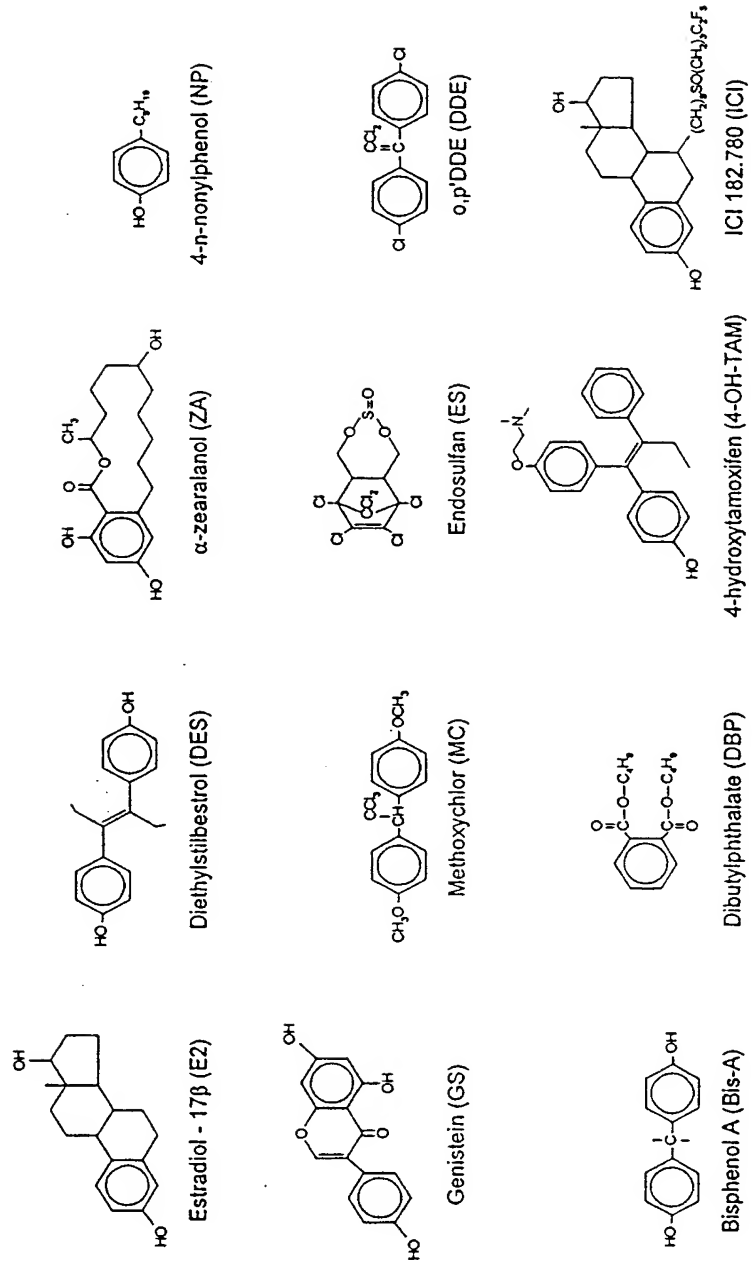
39. Use according to claims 37 or 38, wherein the mammal is a human.

40. Use according to claim 39, wherein the mammal is a post-menopausal female.

41. A method for identifying compounds that act on the level of endogenous gene expression through activating nuclear receptors.

42. A method for evaluating the estrogenic activity of a chemical by assaying induction or repression of endogenous estrogen-regulated marker genes in human estrogen-dependent breast cancer cells.

5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Bisphenol A (Bis-A)

Fig. 1

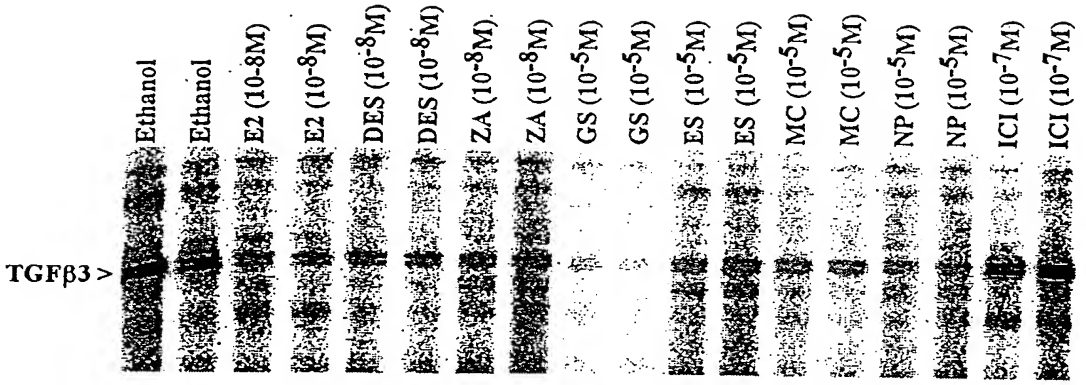
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Fig. 2

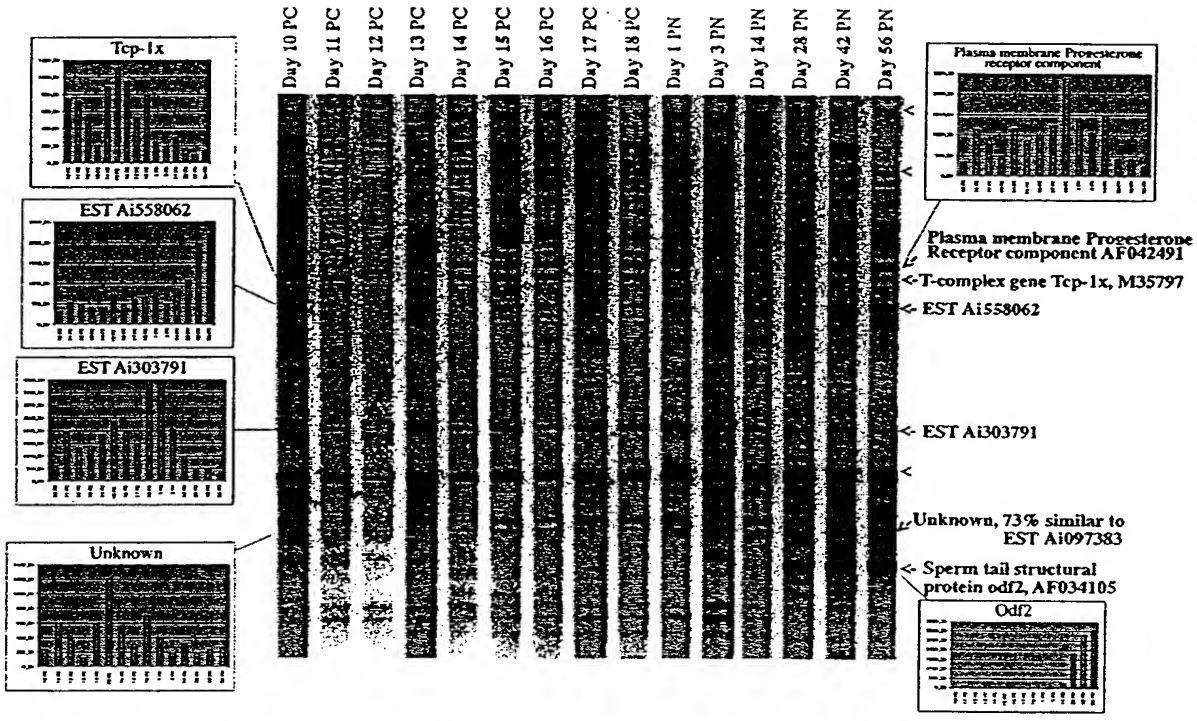
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Fig. 3

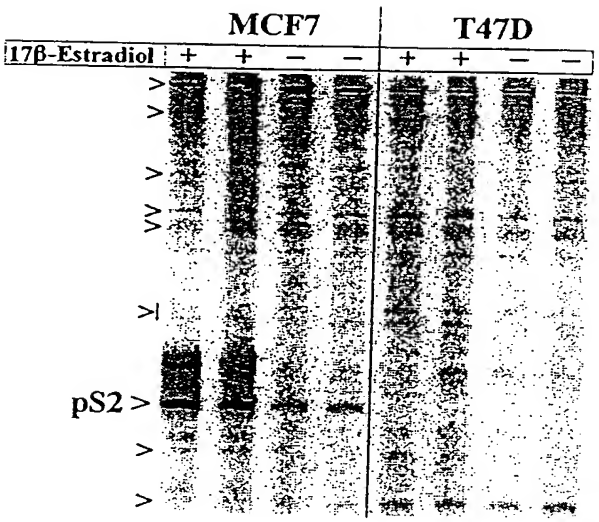

Fig. 4

Fig. 6 A

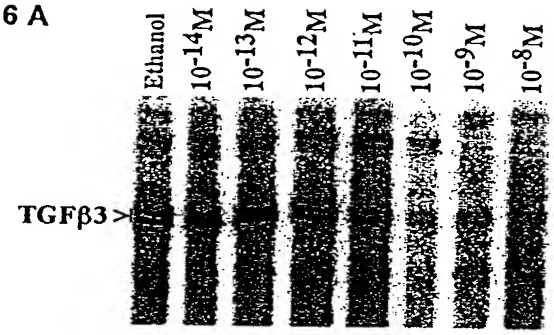

Fig. 6 B

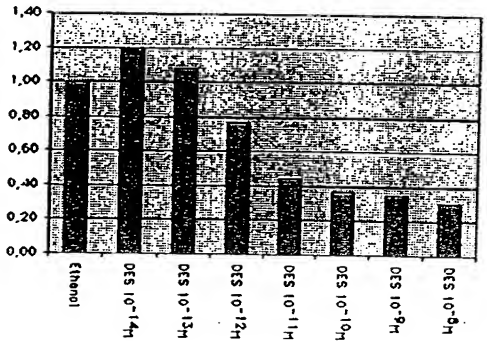

Fig. 6

Fig. 7 A

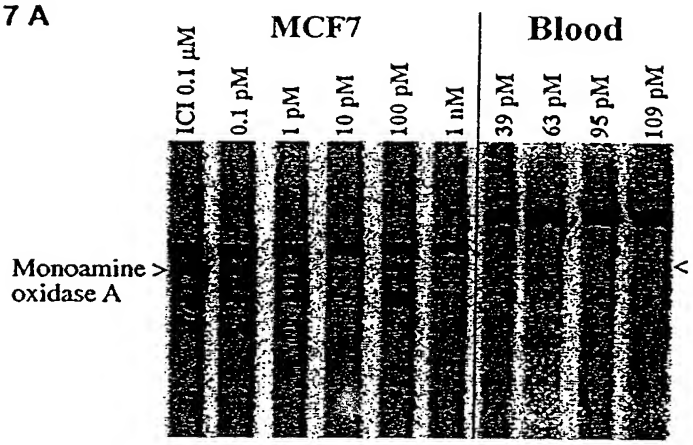

Fig. 7 B

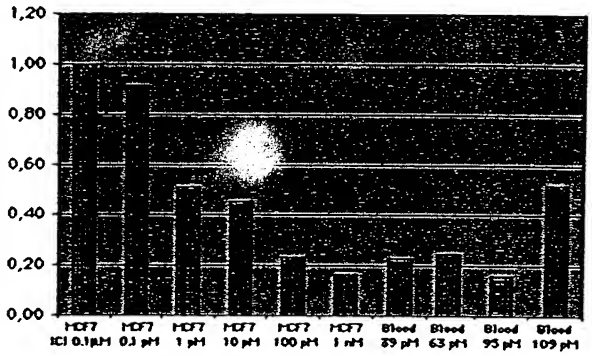

Fig. 7

Fig. 8 A

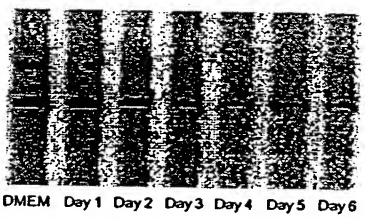

Fig. 8 B

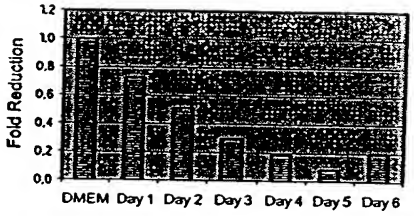

Fig. 8

Fig. 9 A

Fig. 9 B

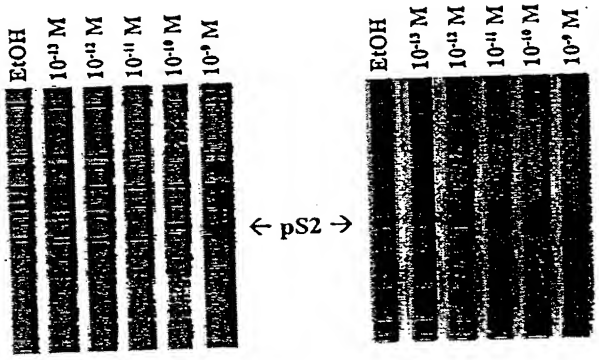

Fig. 9

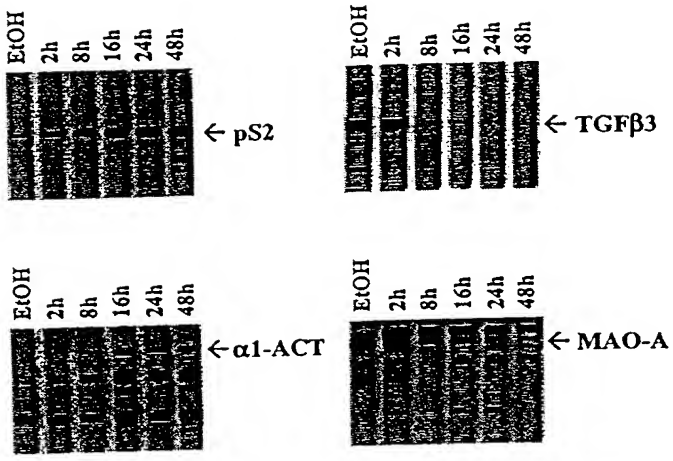
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Fig. 10

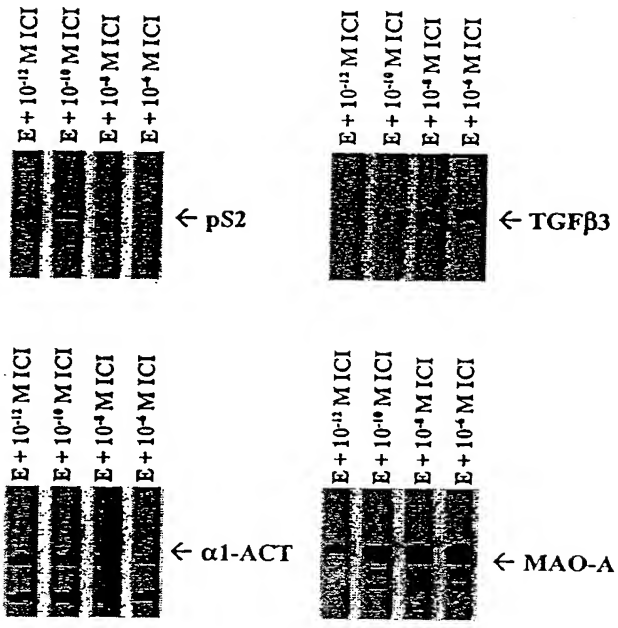
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Fig. 11

Fig. 12 A

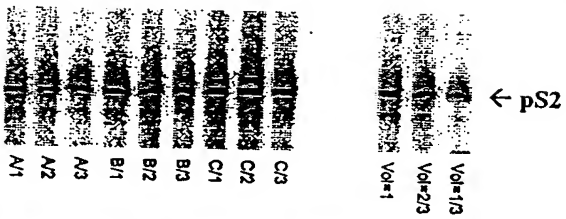

Fig. 12 B

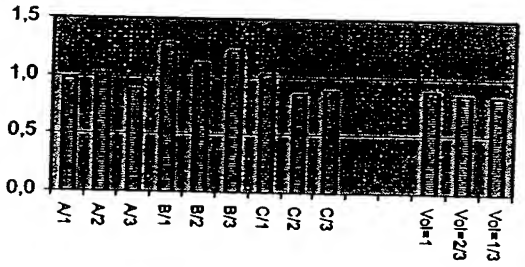

Fig. 12

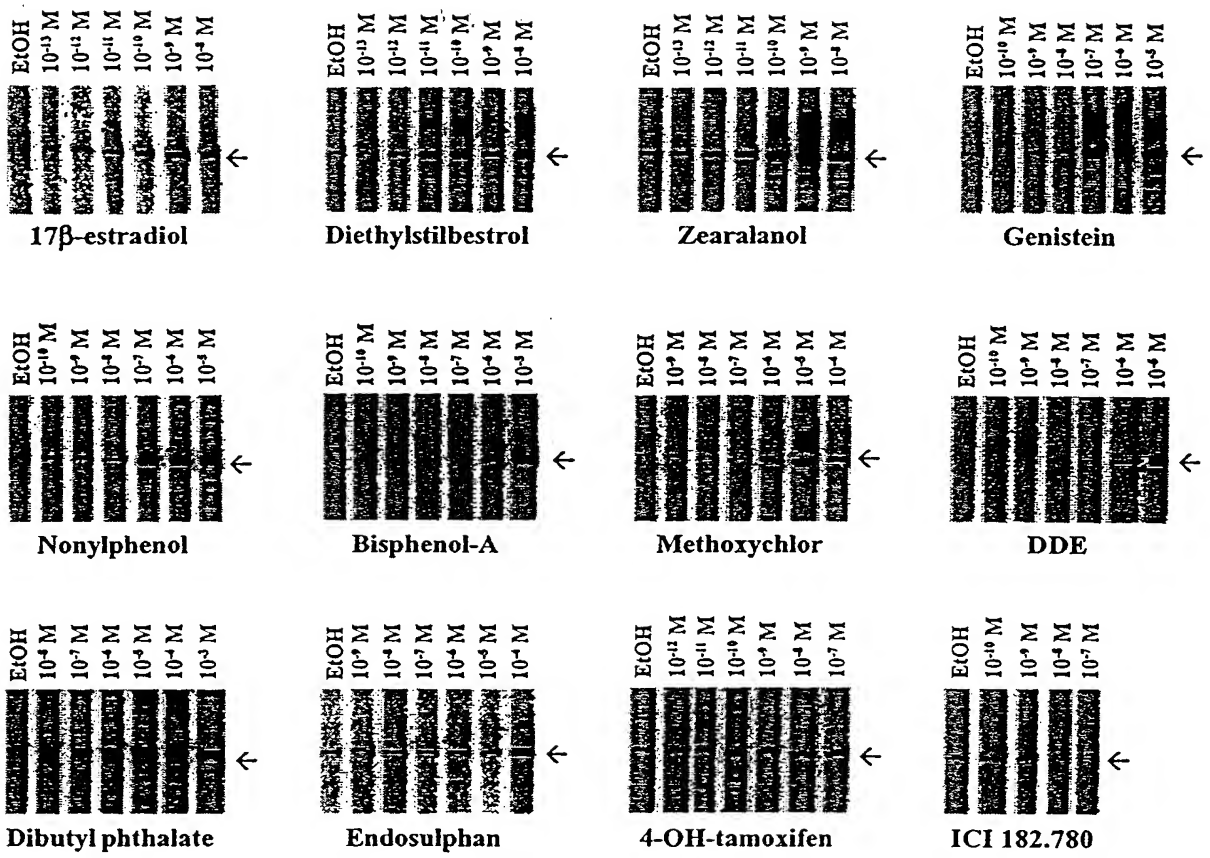
EtOH
10⁻¹³ M
10⁻¹² M
10⁻¹¹ M
10⁻¹⁰ M
10⁻⁹ M
10⁻⁸ M
17β-estradiol
Diethylstilbestrol
Zearalanol
Genistein
10⁻¹⁰ M
10⁻⁹ M
10⁻⁸ M
10⁻⁷ M
10⁻⁶ M
10⁻⁵ M
Nonylphenol
Bisphenol-A
Methoxychlor
DDE
10⁻⁸ M
10⁻⁷ M
10⁻⁶ M
10⁻⁵ M
10⁻⁴ M
10⁻³ M
Dibutyl phthalate
Endosulphan
10⁻¹² M
10⁻¹¹ M
4-OH-tamoxifen
ICI 182.780

Fig. 13

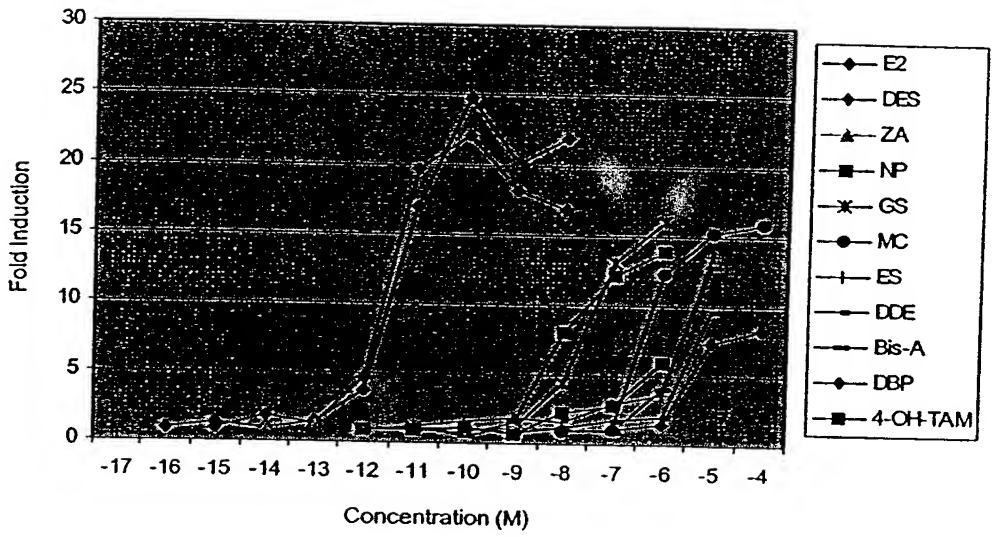
30
25
20
15
10
5
0
Fold Induction
-17 -16 -15 -14 -13 -12 -11 -10 -9 -8 -7 -6 -5 -4
Concentration (M)
E2
DES
ZA
NP
GS
MC
ES
DDE
Bis-A
DBP
4-OH-TAM

Fig. 14

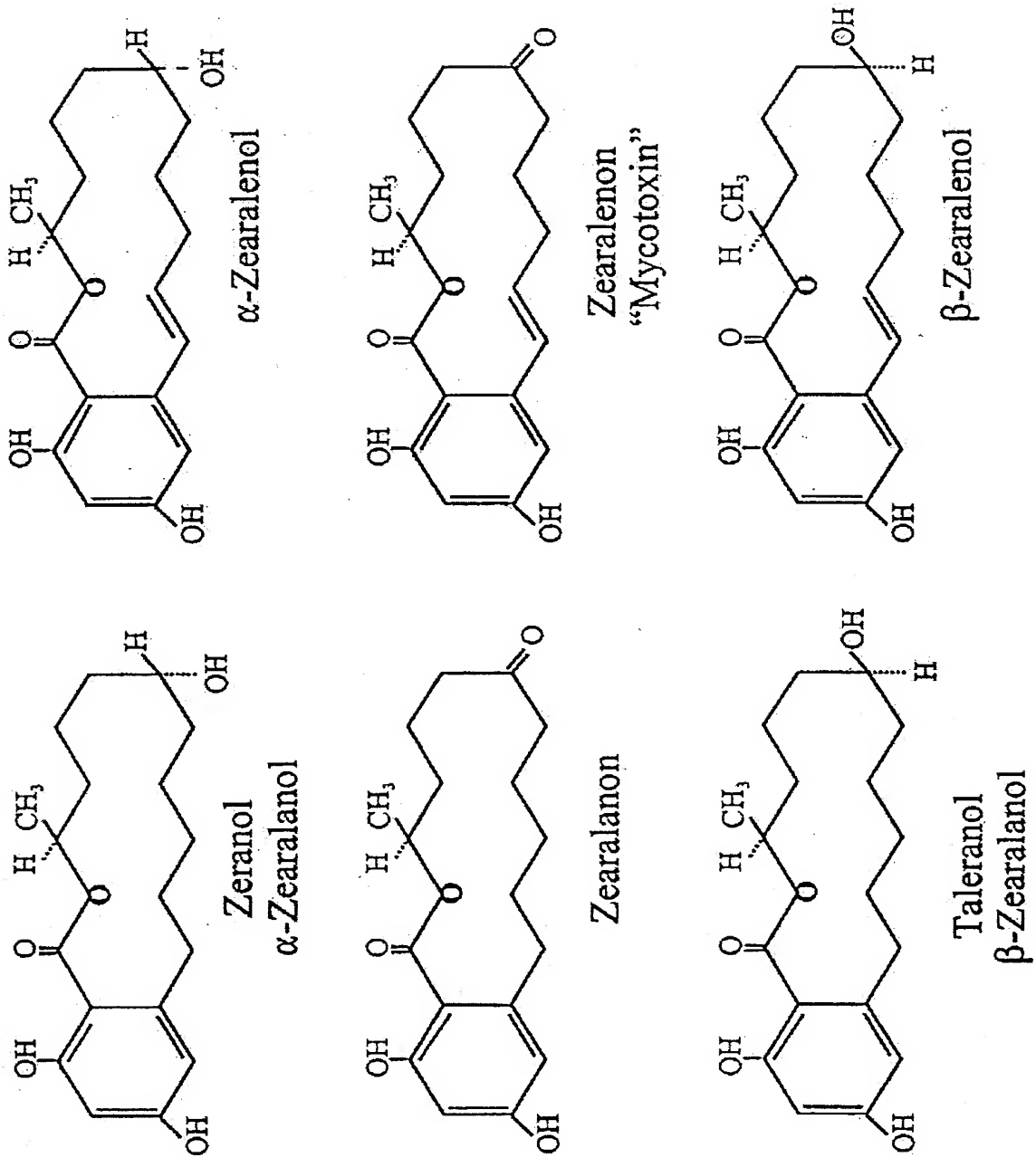
α-Zearalenol
Zearalenon
"Mycotoxin"
β-Zearalenol
Zeranol
α-Zearalanol
Zearalanon
Taleranol
β-Zearalanol

Fig. 15

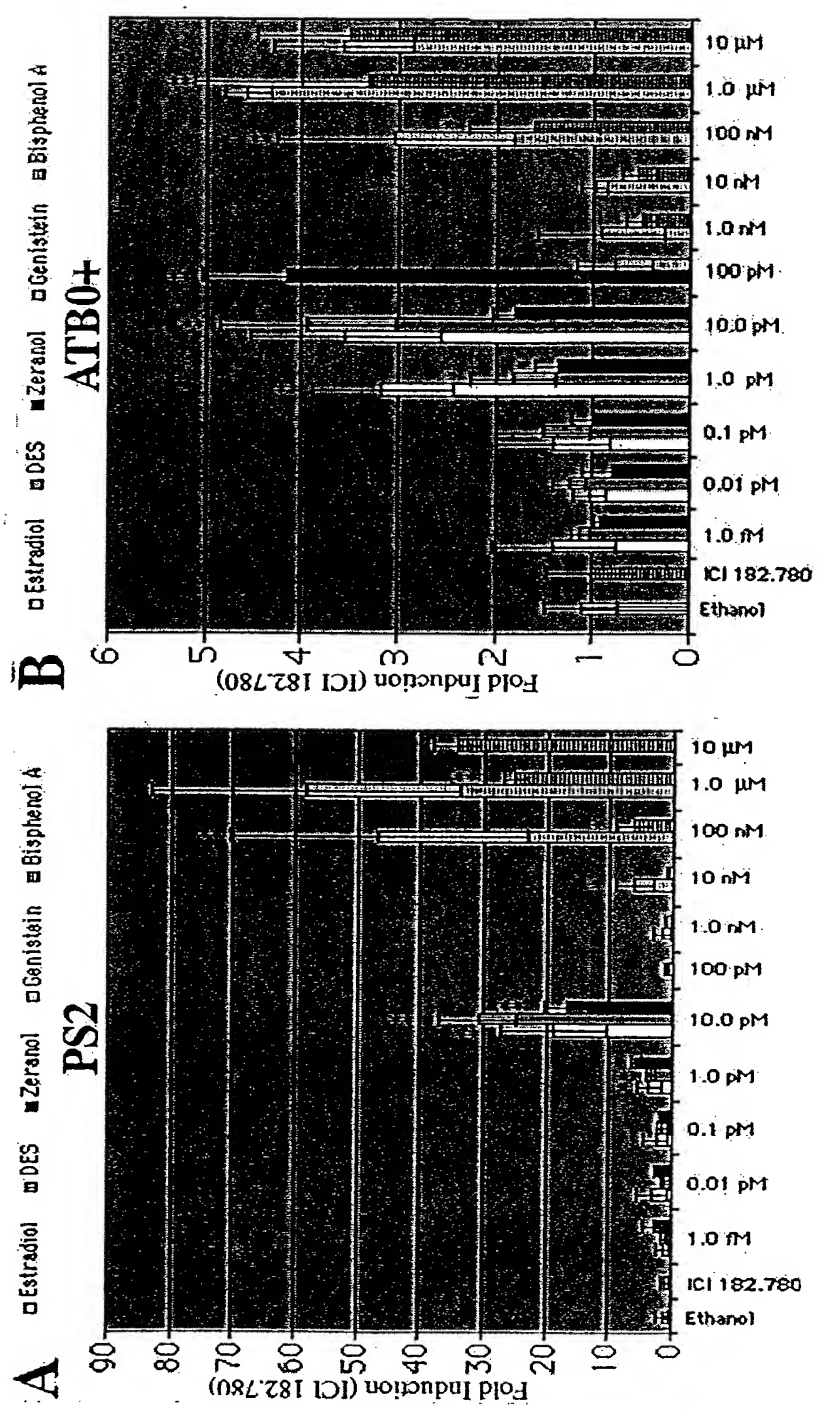
A
PS2
B
ATB0+
Estradiol
DES
Zeranol
Genistein
Bisphenol A
Fold Induction (ICI 182,780)
10 µM
1.0 µM
100 nM
10 nM
1.0 nM
100 pM
10.0 pM
1.0 pM
0.1 pM
0.01 pM
1.0 fM
ICI 182.780
Ethanol

Fig. 16

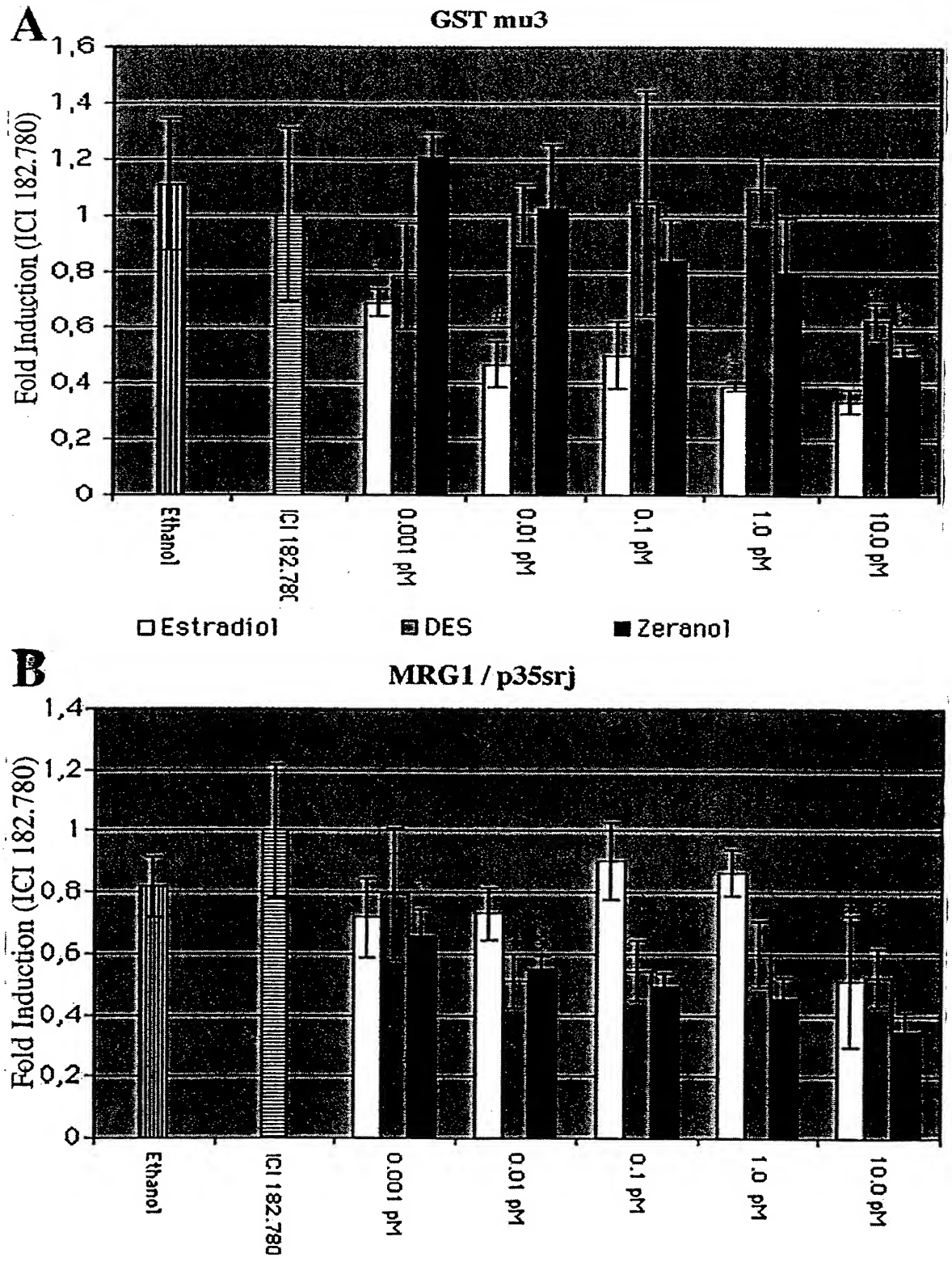
A
GST mu3
Fold Induction (ICI 182.780)
1,6
1,4
1,2
1
0,8
0,6
0,4
0,2
0
Ethanol
ICI 182.780
0.001 pM
0.01 pM
0.1 pM
1.0 pM
10.0 pM
Estradiol
DES
Zeranol
B
MRG1 / p35srj
Fold Induction (ICI 182.780)
1,4
1,2
1
0,8
0,6
0,4
0,2
0
Ethanol
ICI 182.780
0.001 pM
0.01 pM
0.1 pM
1.0 pM
10.0 pM

Fig. 17

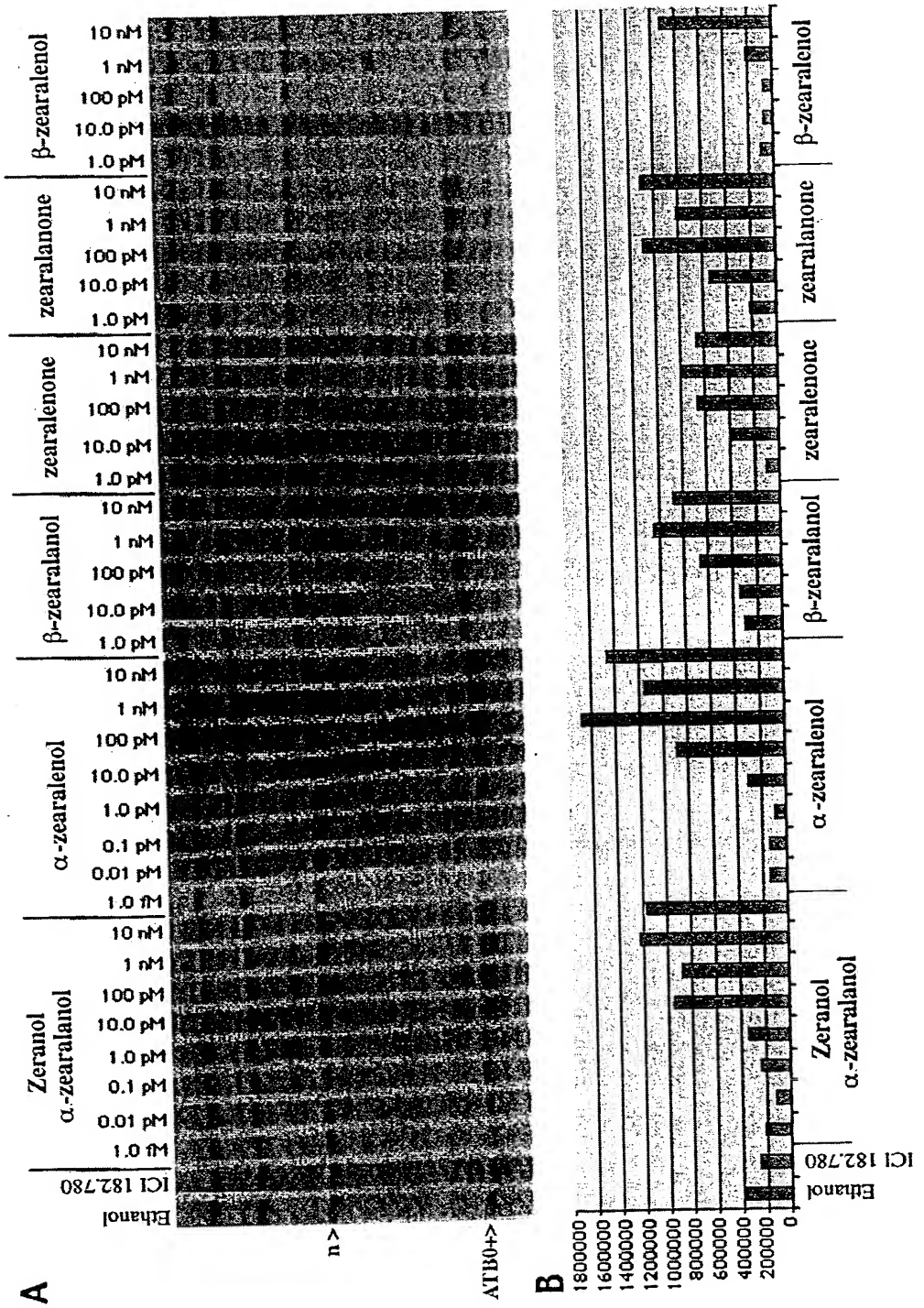
A
Ethanol
ICI 182,780
Zeranol
α-zearalanol
10 nM
1 nM
100 pM
10.0 pM
1.0 pM
0.1 pM
0.01 pM
1.0 fM
α-zearalenol
β-zearalanol
zearalenone
zearalanone
β-zearalenol
n>
ATB0+>
B
1800000
1600000
1400000
1200000
1000000
800000
600000
400000
200000
0

Fig. 18 A and B

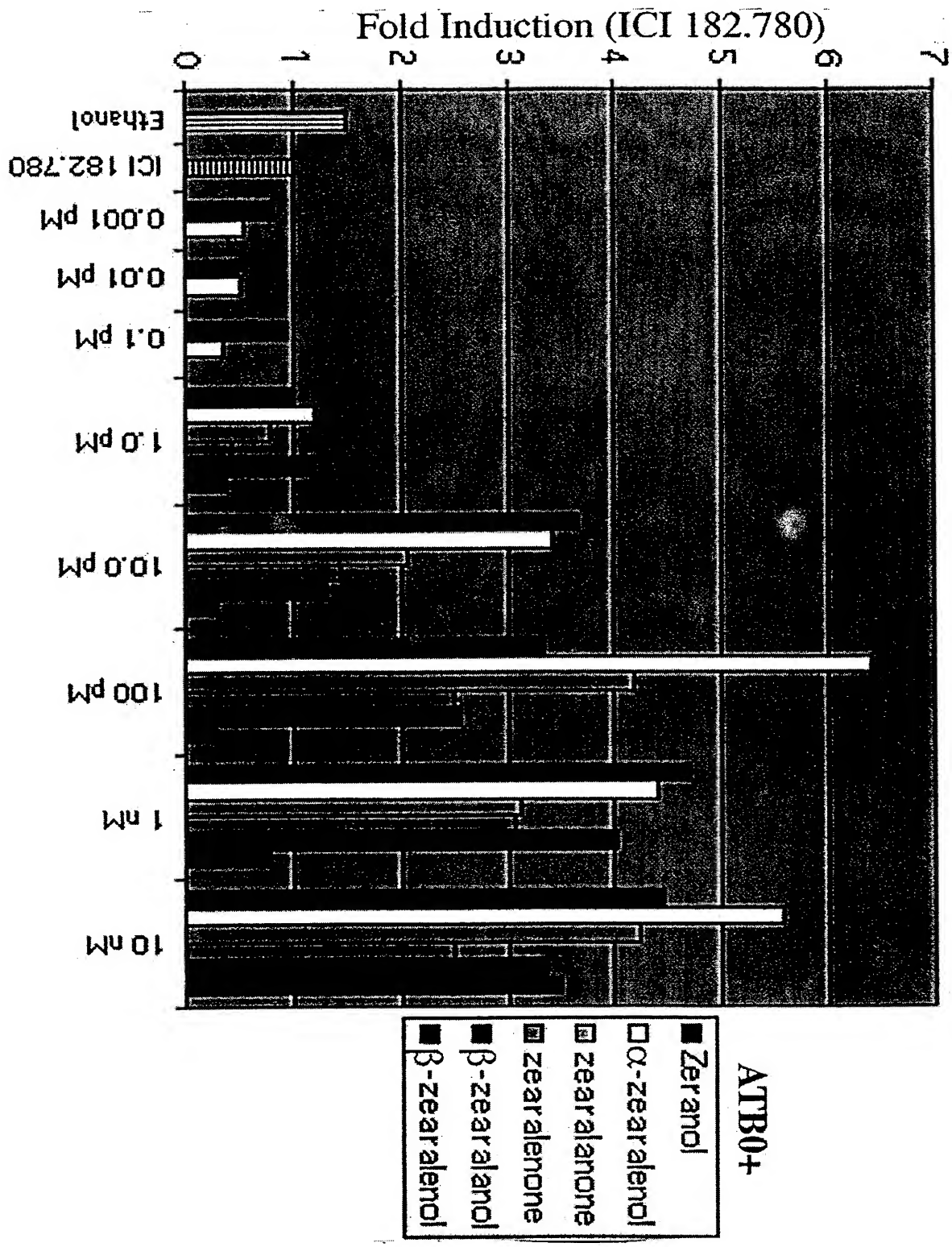
Fold Induction (ICI 182.780)
0
1
2
3
4
5
6
7
Ethanol
ICI 182.780
0.001 pM
0.01 pM
0.1 pM
1.0 pM
10.0 pM
100 pM
1 nM
10 nM
Zerenol
α-zearalenol
zearalanone
zearalenone
β-zearalanol
β-zearalenol
ATB0+

Fig. 18 C